The role of socio-sexual cues in sheep reproduction

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ABSTRACT

Exposure of previously isolated anoestrous ewes to a ram induces an almost instantaneous rise in luteinising hormone (LH) pulse frequency. This physiological response, a phenomenon coined 'the ram effect' is commonly sufficient to override the seasonal suppression of the hypothalamic-pituitary axis and induce a synchronous first ovulation.

The objective of the first series of experiments in this thesis was to develop a non-pharmacological method of oestrus synchronisation, using socio-sexual cues, for natural mating of mule ewes during the breeding season. Initially two experiments were conducted to investigate the effect of short-term fence line and vasectomised ram exposure repeated every 17 days on three occasions during the transition into the breeding season. Ewes repeatedly exposed to the ram had a significantly compacted mating period compared to ewes maintained in isolation from rams prior to mating. This compaction persisted through to lambing with no significant negative effect on litter size. Artificially inseminated ewes synchronised using the above method of ram synchronisation had higher conception rates than progestagen synchronised ewes.

The second objective was to compare the efficacy of different durations and frequencies of ram exposures as methods of oestrus synchronisation. Ewes maintained continuously with rams over the pre-mating period had a more compacted mating and lambing period than ewes exposed intermittently to rams.

Maiden ewes typically show a poorer level of reproductive competence than adult ewes. Similarly maiden ewes induced to ovulate using the ram effect have been found to have a lower ovulatory response. The next objective of this thesis was to determine if pre-exposure to the ram during anoestrus or the breeding season would modulate the hormonal and behavioural responses of maiden ewes when re-introduced to rams during the breeding season or anoestrus. There was no major effect of prior experience of the ram on any parameters of the LH response to ram introduction. However ewes with prior ram experience did have more positive interactions with the rams and demonstrated more ram seeking behaviour.

Incorporation of socio-sexual cues with artificial methods of reproductive control has to date been restricted to ram exposure post progestagen sponge withdrawal. Therefore the final objective of this thesis was to investigate the effect of ram exposure towards the end of a progestagen synchronisation protocol on ewe fertility. There was no significant difference in conception rates between ram exposed and control ewes, however ram exposed ewes had a significant depression in mean litter size due to a greater number of ewes having single lambs.

The studies in this thesis show a robust and repeatable endocrine response to ram introduction in mule ewes exposed to the ram during the transition between anoestrus and the breeding season. The potency of the socio-sexual cues from the ram permits modification of the distribution of oestrus within randomly cycling ewes. The findings in this thesis highlight the potential for application and development of pre-mating strategies using socio-sexual cues within seasonal breeds of sheep.

DECLARATION

I certify that no part of the material offered has been previously submitted for a degree or other qualification in this or any other university.

Penelope Hawken

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1. GENERAL INTRODUCTION

Reproduction in the ewe involves a cascade of endocrine and neural events that are heavily influenced by season, nutrition and social interactions. From an evolutionary perspective it is critical to coordinate reproductive activity between the male and female and to relate the timing of reproductive activity with the supply of food and water to permit successful conception and species survival.

1.1 THE OESTROUS CYCLE

The oestrous cycle is a self perpetuating series of events that are under neuroendocrine control (Figure 1.1). The beginning of the oestrous cycle is initiated by ovulation of a mature Graffian follicle that under the continuing influence of luteinising hormone forms a corpus luteum. The corpus luteum releases progesterone that maintains low circulating concentrations of LH and prevents ovulation of any developing follicles. This period of progesterone dominance is termed the luteal phase, where oestradiol and progesterone act synergistically to maintain a low level of LH pulse frequency (Martin *et al.*, 1983a). An increase in prostaglandin $F_{2\alpha}$ causes regression of the corpus luteum and the onset of the follicular phase (Figure 1.1) where the endocrine milieu is primarily dominated by oestradiol (Review; Clarke, 1984). Due to the relatively weak inhibition of LH by oestradiol during the breeding season, there is a progressive increase in LH pulse frequency (Review; Karsch, 1980). Figure 1.1 shows the progressive increase in oestradiol and LH relative to the declining concentrations of progesterone induced by regression of the corpus luteum. The progressive increase in LH and oestradiol from the developing follicle eventually reaches a critical threshold and the initiation of an LH surge that leads to ovulation of the follicle and resumption of another cycle.

1.1.1 NEUROENDOCRINE CONTROL

The control of the oestrous cycle is exerted by interplay between hormones synthesised in the hypothalamus, anterior pituitary gland and the gonads as illustrated in Figure 1.2.

1.1.1.1 GONADOTROPHIN RELEASING HORMONE

Gonadotrophin releasing hormone (GnRH) is synthesised in the neurons of the hypothalamus and is released into the median eminence for transport by the hypophyseal portal system to the infundibulum (Sherwood *et al.*, 1976). GnRH

release is pulsatile and Figure 1.2 shows the direct relationship between pulses of GnRH and pulses of luteinising hormone (LH).

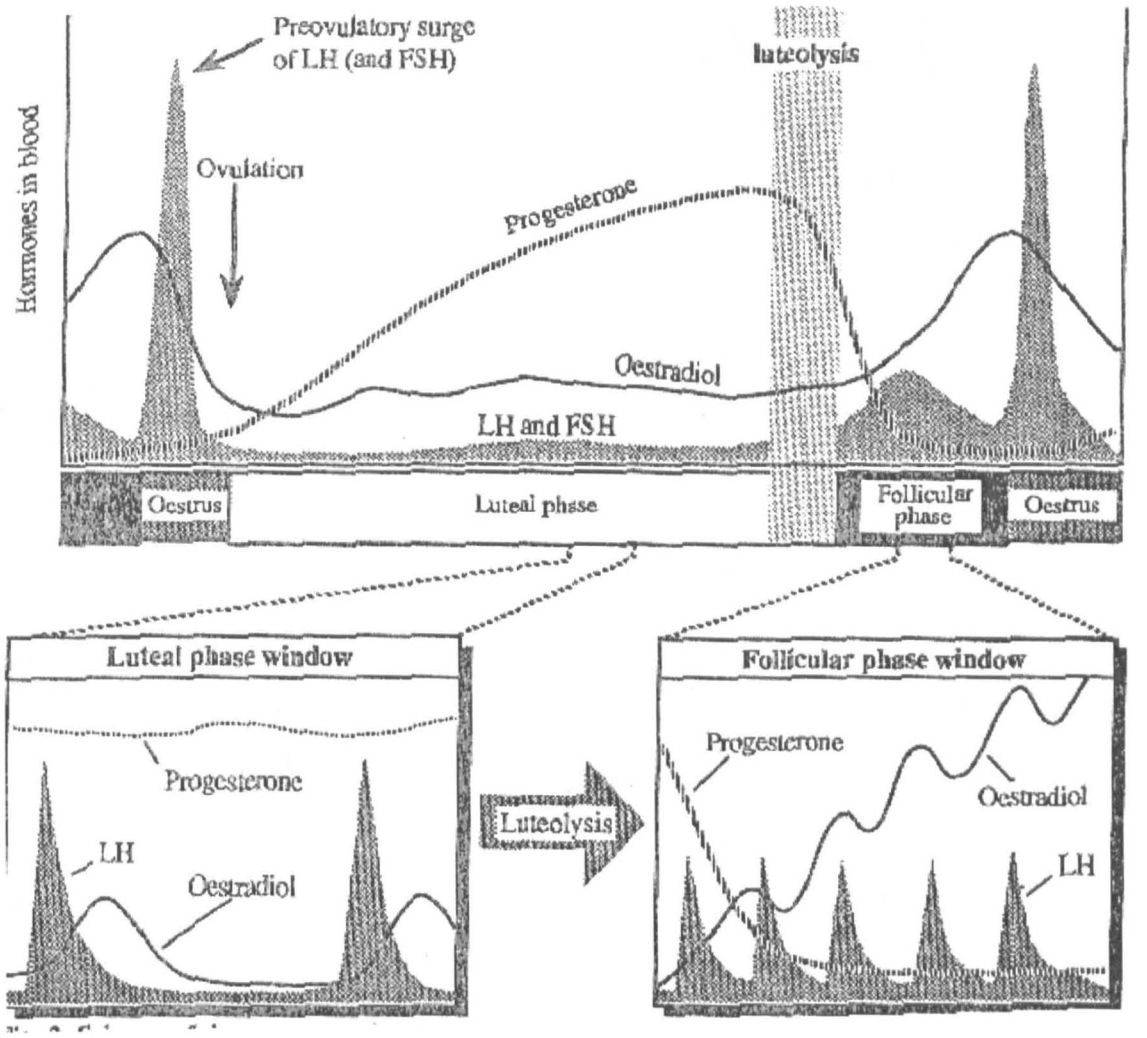


Figure 1.1 Schematic representation of the endocrine events during the oestrous cycle of the ewe (Martin and Thomas, 1990)

1.1.1.2 **LUTEINISING HORMONE**

Luteinising hormone (LH) is released from the anterior pituitary gland and is critical to follicular development and ovulation and is so called, as it is responsible for luteinsiation of the ovulated follicle and formation of a functional corpus luteum. At all stages of the oestrous cycle, a pulse of LH stimulates synthesis and secretion of oestradiol from the granulosa cells of mature Graffian follicles (review; Baird and McNeilly, 1981).

During the luteal phase, LH release is suppressed by a complex interplay between progesterone and oestradiol (Karsch *et al.,* 1977) and LH pulse frequency is low and pulse amplitude high (Martin, 1984). At luteolysis the declining levels of progesterone are associated with an increase in LH pulse frequency as oestradiol alone is a poor suppressor of LH release (Martin, 1984). During the follicular phase oestradiol alone is unable to suppress LH release, thus resulting in an increase in high frequency, low amplitude pulses of LH. The cumulative increase in LH and oestradiol concentrations eventually reaches a threshold and stimulates the onset of the LH surge. The LH surge is composed of frequent, low amplitude pulses of LH (Baird, 1978; Martin *et al.,* 1987)

1.1.1.3 FOLLICLE STIMULATING HORMONE

Follicle stimulating hormone (FSH) is released from the anterior pituitary gland and is critical to follicle development. FSH concentrations fluctuate over the oestrous cycle in a similar way to LH however release is not pulsatile or as markedly constrained by progesterone (Findlay and Clarke, 1987). FSH release is controlled almost entirely by hormones produced from the developing antral follicles, namely oestradiol and inhibin, which act synergistically to suppress FSH release at the level of the anterior pituitary gland (Martin *et al.,* 1988).

1.1.1.4 OESTRADIOL

Oestradiol is synthesised from the thecal cells of the developing follicle and is involved in regulation of follicle development within the follicular wave (Scaramuzzi *et al.,* 1993). Oestradiol production increases with the size and developmental stage of the follicle and acts synergistically with inhibin to suppress FSH below that required for development of smaller, gonadotrophin dependent follicles (Scaramuzzi *et al.,* 1993).

1.1.1.5 INHIBIN

Inhibin is synthesised by the granulosa cells of gonadotrophin independent and ovulatory follicles and as outlined above directly inhibits secretion of FSH. Immediately after the pre-ovulatory surge of LH, inhibin levels decrease which coincides directly with a surge of FSH (Findlay *et al.,* 1991).

1.1.2 OESTROUS BEHAVIOUR

Expression of oestrous behaviour requires a period of progesterone priming to permit elicitation of oestrogen induced oestrous behaviour (Robinson, 1954)

1.1.2.1 NEURAL CONTROL OF OESTROUS BEHAVIOUR

Recent work (Fabre-Nys *et al.*, 2003) has highlighted the role of a number of neurotransmitters in the medial basal hypothalamus critical to the expression of sexual behaviour in the ewe, specifically mesolimbic dopamine and noradrenalin. Dopamine has been shown to have a biphasic role in optimising specifically the receptivity of the ewe to the ram. Levels of dopamine are elevated for the duration of the declining progesterone phase associated with the regressing corpus luteum that has the effect of placing a hypothetical brake on the neural processes occurring within the medial basal hypothalamus. The increase in oestradiol that occurs with the growth and development of the dominant follicle induces a sharp drop in the levels of dopamine that subsequently remain low, and is ensued by an increase in levels of noradrenalin and rapid expression of behavioural oestrus (Fabre-Nys *et al.*, 2003). The function of this stimulation/suppression pattern of dopamine release is to prime the neural mechanism and result in optimum receptivity of the ewe to the ram. If the endocrine pattern of events alters, such as during the onset of the breeding season where the first cycle of the breeding season is not preceded by a sustained period of elevated progesterone (Karsch *et al.*, 1984), this pattern of dopamine release is affected and behavioural oestrus does not occur and the receptivity of the ewe to the ram is low (Fabre-Nys *et al.*, 2003).

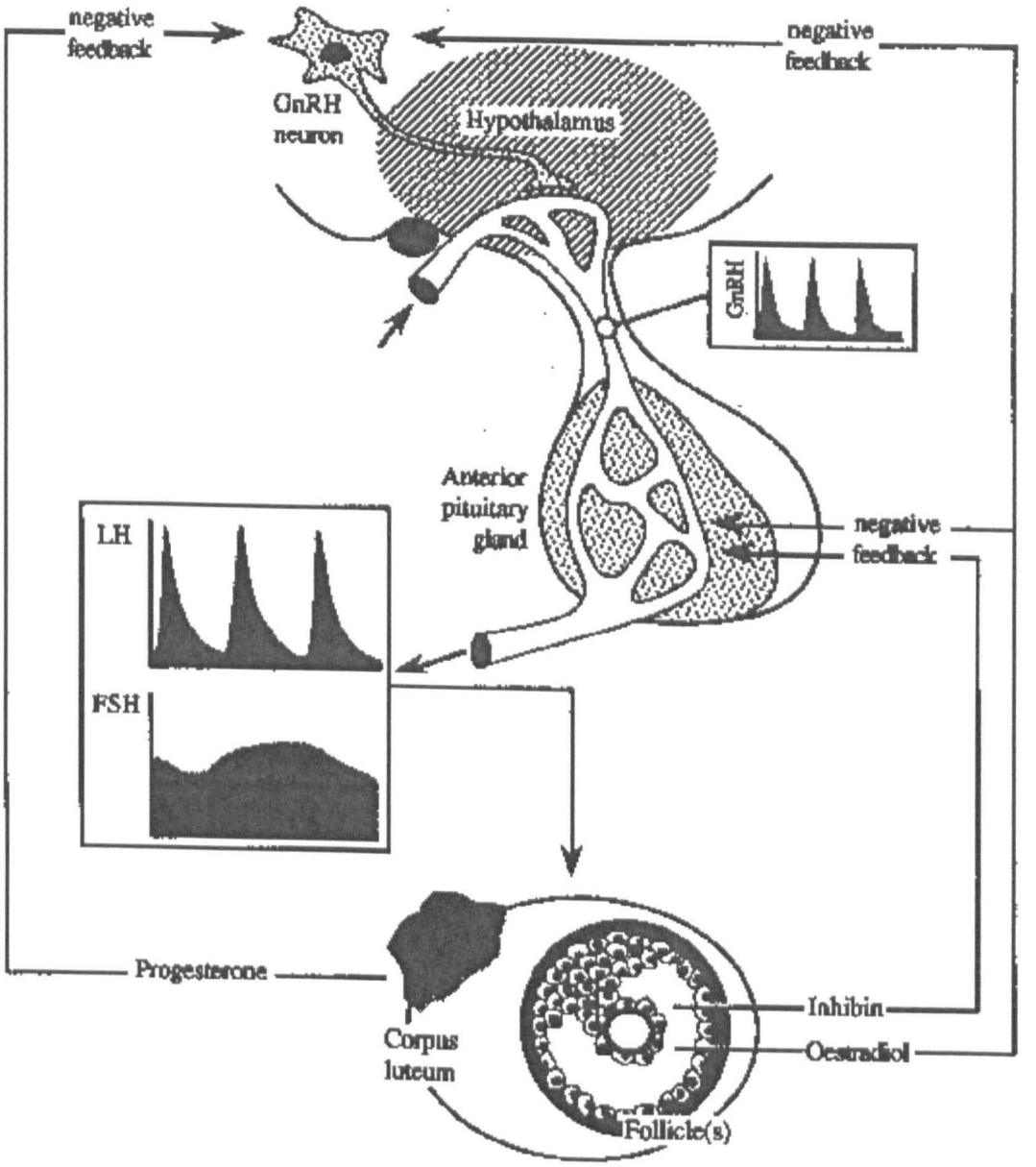


Figure 1.2 Schematic representation of the endocrine links between the hypothalamus, pituitary gland and ovary. Important points to note are the pulsatile nature of release of GnRH from the hypothalamus that stimulates pulsatile release of LH from the anterior pituitary gland. This is in contrast to GnRH stimulated release of FSH that is not pulsatile. (Thiery and Martin, 1991)

1.2 SEASONALITY

Seasonal breeding has been termed natures contraceptive as it coordinates the onset of reproductive activity with the projected time of plentiful food and water supply (Lincoln and Short, 1980). The majority of sheep breeds are anoestrus for at least some proportion of the year (Rekwot *et al.,* 2001) with the degree and depth of seasonality dependent upon breed and climate. Studies have found that within sheep native to temperate climates, photoperiod plays the preponderant role in controlling the onset and succession of reproductive activity (Karsch *et al.,* 1984). This greater dependency on photoperiod corresponds with a greater 'depth of anoestrus' in temperate breeds of sheep. In contrast, breeds of sheep native to more tropical climates have a short and light anoestrous period with food supply and temperature playing the major role in dictating the distribution of reproductive activity (Karsch, 1984).

1.2.1 ANNUAL PATTERN OF REPRODUCTIVE ACTIVITY

The breeding season of the ewe is characterised by spontaneous behavioural oestrus and ovulation, on average every 17 days. In the absence of fertilisation and pregnancy the pattern of events outlined in Figure 1.1 occurs recurrently until the cessation of breeding activity and the onset of the anoestrous period. The transition into anoestrus occurs gradually with an increased sensitivity of the GnRH pulse generator to the negative effects of oestradiol (Karsch, 1984) that will be outlined in detail in Chapter 1.2.2. The anoestrous period is characterised by low LH pulse frequency (one pulse every 8-12 hours; Karsch *et al.,* 1984) which is a marked depression even compared to pulse frequency during the heavily progesterone influenced mid luteal phase (one pulse every 3-4 hours; Yuthasastrakol *et al.,* 1977). During anoestrus, follicles continue to grow and develop to a similar size observed in the breeding season (Review; Rawlings *et al* 2003). However the potency of oestradiol to suppress LH release is markedly enhanced (Legan *et al.,* 1977) and thus prevents ovulation

The period of transition from anoestrus to the breeding season commences between 1 and 4 weeks before the onset of the breeding season (I'Anson and Legan, 1988) and is characterised by fluctuating sensitivity of the hypothalamic-hypophyseal axis to oestradiol resulting in shifts in LH pulse frequency (Karsch *et al.,* 1984). These spontaneous, episodic elevations in LH concentrations last only 1-3 days and are

typically followed by a transient rise in progesterone (I'Anson and Legan, 1988) thought to synchronise follicular dynamics and ensure a normal luteal phase at the onset of cyclicity (Legan *et al.*, 1985; Ravindra and Rawlings, 1995). Short oestrous cycles (5-6 days) are common at the onset of the breeding season and typically occur without oestrus (Review; Bartleswki *et al.*, 1999).

1.2.2 PHOTO-NEUROENDOCRINE CONTROL

Photoperiod controls the onset and cessation of breeding activity through a gradual decrease or increase of the sensitivity of the hypothalamic-hypophyseal axis to the negative effects of oestradiol (Karsch *et al.*, 1980). During the transition into anoestrus the regression of the corpus luteum of the last oestrous cycle induces the characteristic increase in concentrations of LH. However the increase in LH is constrained by the accompanying increase in oestradiol from the developing follicles thus preventing ovulation (Karsch *et al.*, 1980). The negative effects of oestradiol on LH release during anoestrus are mediated at the level of the hypothalamus by a reduction in GnRH pulse frequency, rather than a depression in pituitary responsiveness to GnRH (Karsch *et al.*, 1987; 1993).

The sensitivity of the hypothalamic-hypophyseal axis to the negative effects of oestradiol is dictated by photoperiod. The mechanism behind the mediation of the photic stimuli to the hypothalamus process is clearly represented in Figure 1.3. Light is detected by photoreceptors on the retina and relayed to the suprachiasmatic nuclei of the hypothalamus (Karsch *et al.*, 1984). The neural input is converted into a hormonal signal at the level of the pineal gland with the duration of melatonin elevation directly proportional to night length. Therefore as sheep are short day breeders, diminished melatonin that is indicative of a short night length, sensitises the GnRH pulse generator to the negative effects of oestradiol thus suppressing GnRH release (Figure 1.3). In contrast extended melatonin release stimulates the GnRH pulse generator and desensitises it to the negative effects of oestradiol thus permitting an increase in GnRH pulsatility (Karsch *et al.*, 1984). However as outlined above, the transition in the sensitivity of the hypothalamic hypophyseal axis to the suppressive effect of oestradiol is not an acute change (Karsch *et al.*, 1984).

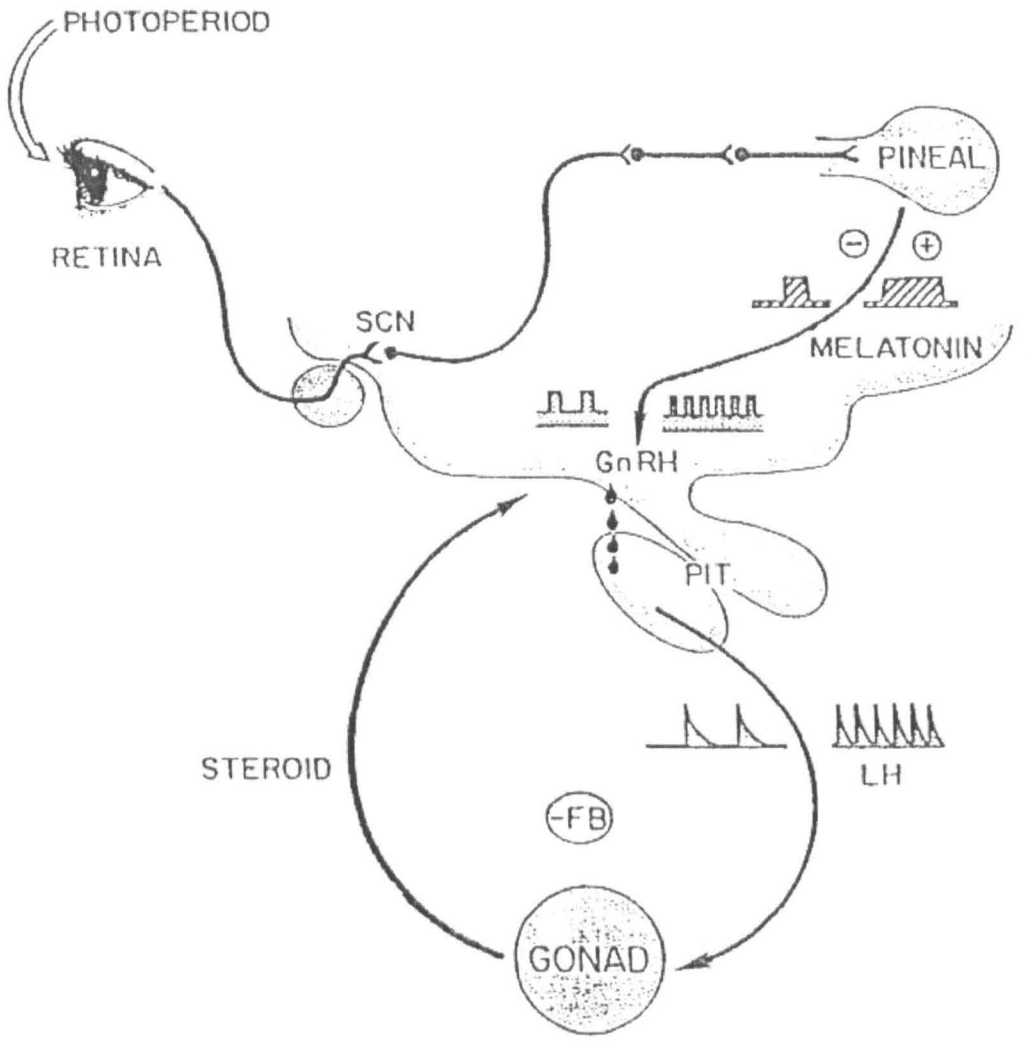


Figure 1.3 Schematic representation of the model for neuroendocrine control of control of oestrous activity and inactivity during the anoestrus and breeding season (Karsch *et al.,* 1984). Abbreviations explained: -

- SCN represents the suprachiasmatic nuclei
- + / - MELATONIN indicates inhibitory or inductive action of melatonin on the hypothalamic-hypophyseal axis
- –FB indicates the negative feedback between ovarian steroids and the hypothalamic-hypophyseal axis.

Although photoperiod is critical in dictating the pattern of reproductive activity, it can also be modulated by other external factors (Karsch, 1984). Introduction of a ram to anoestrous ewes stimulates an increase in LH pulse frequency that overrides or bypasses this photoneuroendocrine control of reproductive activity to induce an LH surge and ovulation (Review; Martin *et al.* 1986).

1.3 PHARMACOLOGICAL METHODS OF MANIPULATING OESTROUS CYCLES IN THE EWE

Pharmacological control of oestrus using synthetic progestagens was developed during the 1950s and permits synchronisation of breeding both during the breeding season and anoestrus (Robinson 1954). Intravaginal pessaries are commonly used to synchronise the oestrous cycles of sheep, as the pessary mimics the action of a corpus luteum by providing an artificial source of progesterone that is sufficient to suppress gonadotrophin production (Robinson 1974). Removal of the pessary removes the progestagen block and induces synchronous re-instatement of gonadotrophin release and subsequent ovulation in treated ewes. Artificial progestagen synchronisation is frequently associated with administration of equine chorionic gonadotrophin (eCG) that is also known as pregnant mare serum (PMSG). The purpose of these substances is to improve ovulation rate and synchrony at the synchronised oestrus (Boscos *et al.*, 2002).

1.4 NON-PHARMACOLOGICAL METHODS OF MANIPULATING OESTROUS CYCLES IN THE EWE

Ram introduction to anoestrous ewes elicits an almost instantaneous increase in LH pulse frequency, typically within 2-4 minutes of ram introduction as shown in Figure 1.4 (Martin *et al.*, 1986; Gelez *et al.*, 2004a). This physiological response was first identified by Underwood *et al.*, (1944) and was termed the 'ram effect'. Under certain physiological states, this endocrine response is sufficient to overcome the seasonal suppression of the hypothalamic-pituitary axis and induce an LH surge and ovulation. Within responsive breeds of sheep, the subsequent distribution of oestrus and maintenance of cyclic activity is relatively predictable and permits out of season breeding (Reviews; Martin *et al.*, 1986; Walkden-Brown *et al.*, 1999; Rosa and Bryant 2002). Manipulation of reproductive activity of anoestrous females using oestrous females (Zarco *et al.*, 1995; Nugent and Notter, 1990; O'Callaghan et al., 1994) is a contentious issue and will be discussed fully in Chapter 2.3.2.

Endocrine response of anoestrous females to the ram effect

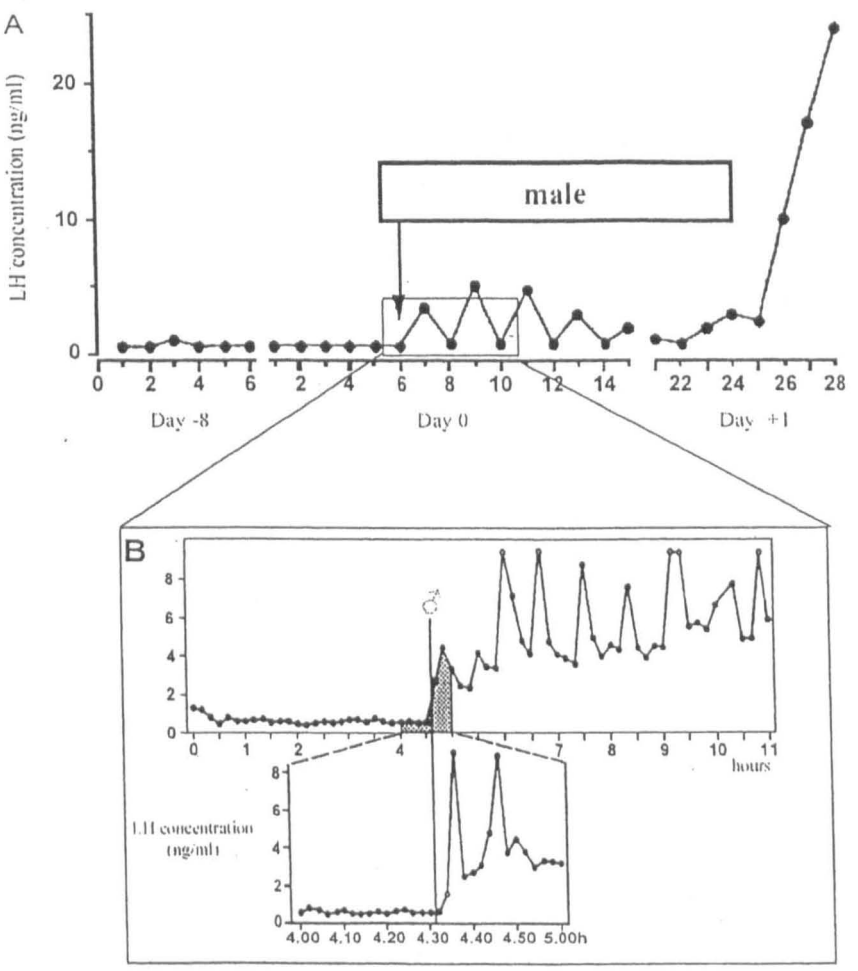


Figure 1.4 Schematic representation of LH release in anoestrous ewes introduced to a ram - The 'ram effect'; Gelez and Fabre-Nys (2004)

1.5 WHY IT IS IMPORTANT TO STUDY NATURAL METHODS OF MANIPULATING REPRODUCTION THAT ARE APPLICABLE TO UK SYSTEMS.

Oldham and Pearce (1984) identified the ram effect as a potential method of controlling sheep breeding to provide farmers with a cheap, reliable and non-pharmacological method of oestrus synchronisation. The ram effect is used widely in sheep production in Australia and New Zealand for breeding of ewes during anoestrus (Review; Martin *et al.,* 2004) and in artificial insemination protocols (Corke, 1980). However in the UK, application of the ram effect is limited to late anoestrus and the magnitude, repeatability and predictability of the response is lower than in less seasonal breeds of sheep. Therefore development of pre-mating strategies using socio-sexual cues remains a relatively untapped resource.

In the absence of modification of the seasonality of British breeds of ewe using melatonin implants (Rekik *et al.,* 1991; Rosa *et al.,* 2000), adoption of management techniques using the ram effect for out of season breeding may never be applicable to UK sheep production. However evidence of an endocrine and ovulatory response to the ram effect late in anoestrus (Rosa et al., 2000; Al-Mauly et al., 1991) infers the potential for developing ram effect strategies that are specifically designed for seasonal breeds of sheep.

The specific importance of development of non-pharmacological strategies for manipulating reproduction is highlighted by the increasing consumer demand for more "clean, green and ethical" farm practices (Martin *et al.,* 2004). Furthermore pharmacological synchronisation procedures are unacceptable in organic production systems (Compendium of organic standards, 2004) therefore preventing the potential for genetic improvement through artificial insemination.

2. REVIEW OF THE RELEVANT LITERATURE

In the wild, ewes and rams will spend the anoestrous period in isolation from each other, which results in an intense period of stimulatory sexual activity at the time of reunification (Shackelton and Shank, 1984). The co-ordination of sexual receptivity between males and females plays a vital role in the reproductive and thus evolutionary success of a species (McClintok, 1983). This is of particular relevance within prey species such as the sheep, where the co-ordination of reproductive activity and thus parturition is a defence mechanism against predators (Walkden Brown *et al.*, 1999).

Pheromones are an airborne chemical substance secreted externally from an animal that result in a specific reaction in another animal, resulting in an endocrine and/or behavioural response (Doty, 1976). Olfactory communication plays a critical role in the coordination and transmission of physiological receptivity between males and females (McClintok, 1983). However though the concept of pheromonal communication is well established in insects and rodents, the relevance of the term 'pheromone' to sensory communication between the ewe and the ram has been questioned. Martin *et al.*, (1986) identified many inconsistencies between the definition of the term pheromone and the stimulus responsible for the ram effect. I have outlined below the definition of the term pheromone and the contradictions identified by Martin *et al.*, (1986) with further modifications based on recent work in the area of the ram effect.

1. *A pheromone should be a single (or very few) characterised compounds*

   Sheep: The ram pheromone has only been partially identified and is composed of substances from both neutral and acid fractions (Cohen-Tannoudji *et al.*, 1994) and is a complex not simple substance (Gelez and Fabre-Nys, 2004).

2. *Evoke responses which are not learned or genetically programmed*

   Sheep: It has not been conclusively determined if learning plays a role in the elicitation of the endocrine response to the ram. Gelez *et al.*, (2004c) found some evidence of a necessity for pre-exposure of sexually naïve maiden ewes to the ram, in order for them to respond to the ram pheromone.

3. *Be specific to one particular action or effect*

   Sheep: This appears to concur to the definition of the pheromone, as it is associated with an increase in LH secretion (Martin *et al.*, 1986).

4. *Not be able to be substituted by any other stimulus*

   Sheep: In sexually experienced ewes, the olfactory stimulus can be completely substituted by exposure to audio, visual and tactile cues from the ram (Cohen-Tannoudji *et al.* 1986).

5. *Be specific for a particular species*

   Sheep: It is not a species-specific response as exposure of anoestrous ewes to buck odour elicits a comparable endocrine response (Knight *et al.*, 1983).

Therefore due to these inconsistencies and in particular the additive (Pearce and Oldham, 1988) and substitutive roles (Cohen-Tannoudji *et al.* 1986) of the non-olfactory stimuli, it is more appropriate to refer to the stimuli responsible for mediating the ram effect as socio-sexual cues.

2.1 THE MALE EFFECT IN ANOESTROUS SHEEP - "THE RAM EFFECT"

2.1.1 THE ENDOCRINE RESPONSE

As stated previously, ram introduction to anoestrous ewes elicits an almost instantaneous increase in LH pulse frequency that in continued ram presence is sustained for at least 12 hours (Martin *et al.*, 1986). Within anoestrous ewes, LH pulse frequency is infrequent due to the suppressive action of oestradiol on the hypothalamic-hypophyseal axis (Legan *et al.*, 1977). However this increase in LH pulse frequency is, in certain breeds of sheep and under certain physiological states, sufficient to override the seasonal suppression of the hypothalamic-hypophyseal axis, resulting in an LH surge and ovulation as shown in Figure 1.4 (Martin *et al.*, 1980). FSH plays a less critical role in the ram effect and changes in circulating concentrations are less rapid (Martin et al., 1983a). Reports of the timing of the LH surge are very variable both within and between breeds (Review; Martin *et al.*, 1986). Studies investigating the ram effect in Merino ewes have reported the timing of the LH surge between 12 ± 3 hours (Pearce *et al.*, 1985) and 37 ± 5 hours (Oldham and Pearce 1983) after ram introduction. More seasonal breeds typically have a later LH surge such as the Prealpes-du-Sud ewes, where timing of the LH surge was recorded

as 40 ± 2 hours (Martin *et al.*, 1985). The period between the LH surge and ovulation is not affected by the presence of the ram and occurs at between 22 and 26 hours after the LH surge (Cumming *et al.*, 1973). Overall however, Martin *et al.*, (1986) suggested that the period available for follicular growth, i.e. that between ram introduction and ovulation, is typically less than 60 hours, which is shorter than during the follicular phase of normal cycling animals.

Therefore in summary most ram affected ewes ovulate between 30 and 72 hours after ram introduction (dependent on breed and depth of seasonality, factors that will be discussed in more detail in Chapter 2.2.1) with the majority of variation from breed and individual differences in time from ram introduction to the LH surge (Martin *et al.*, 1986).

It is reportedly the increase in LH pulse frequency and not pulse amplitude that is the critical factor in determining if ewes ovulate in response to the ram effect (Martin *et al.*, 1986). However it is of interest that LH pulse frequency is not always a reliable indicator of whether ewes ovulate in response to the ram effect. Oldham and Pearce, (1983) found that within ewes ovulating in response to the ram effect, a proportion showed a minimal increase in LH pulse frequency yet ovulated within 72 hours of ram introduction.

2.1.2 LUTEAL FUNCTION AND DISTRIBUTION OF BEHAVIOURAL OESTRUS

Conventional application of the ram effect during anoestrus is associated with a biphasic spread of behavioural oestrus between 18 and 25 days after ram introduction (if ram presence is maintained) as shown in Figure 2.1 (Underwood *et al.*, 1944; Oldham and Martin 1978; Pearce and Oldham, 1984). The first peak of oestrous activity around Day 18 is due to ewes that ovulated and had a full-length oestrous cycle in response to ram introduction. The second peak of oestrous behaviour around Day 25 reflects the luteal activity of ewes having a short luteal phase after the initial ram induced ovulation, followed by a normal length luteal phase (Oldham and Martin, 1978). Premature regression (within 5-6 days of ovulation) of the ram induced corpora lutea occurs within 50-60% of ewes (Martin *et al.*, 1986). The rise in progesterone associated with these short lived corpora lutea is typically insufficient to

prime the system, thus resulting in ovulation but again in the absence of oestrus, with the first expression of behavioural oestrus at the third ovulation relative to ram introduction (Pearce *et al.*, 1985). Within the remaining proportion of ewes having normal length luteal phases, behavioural oestrus occurs at the second ovulation relative to ram introduction (Martin *et al.*, 1986).

Short oestrous cycles are not exclusive to ovulations stimulated by the ram effect and occur at the onset of puberty (Keisler *et al.*, 1983) and the breeding season (Legan *et al.*, 1985). The cause of short cycles in ewes stimulated to ovulate by the ram effect was initially thought to be a function of the period available for follicular growth and the random distribution of follicle development within follicular waves (Martin *et al.*, 1986). This was deduced by the eradication of short cycles when ewes are treated with an artificial progestagen prior to ram introduction and the association of this pre-treatment with a lengthened interval between ram introduction and the LH surge (Martin *et al.*, 1980). Furthermore administration of progestagen treated ewes with GnRH to advance the LH surge to the time typically associated with the ram effect resulted in a similar proportion of ewes having short cycles (Pearce *et al.*, 1985).

However more recent work has shown that hysterectomy eliminates the occurrence of short cycles in ewes stimulated to ovulate with the ram effect thus indicting that it is premature release of uterine $PGF_{2\alpha}$ that is the cause of premature regression of the ram induced corpus luteum (Chemineau *et al.*, 1993; Lassoued *et al.*, 1997). The method in which progesterone mediates this premature action of $PGF_{2\alpha}$ is currently unknown, however Lassoued *et al.*, (1997) proposed a possible effect of progesterone priming that restricts up regulation of uterine oxytocin receptors on Day 5 after ram introduction. Furthermore Beard and Hunter (1994) previously identified the delayed decline in oxytocin receptors in ewes not primed with progesterone as the principle mechanism in premature luteolysis of the corpus luteum,

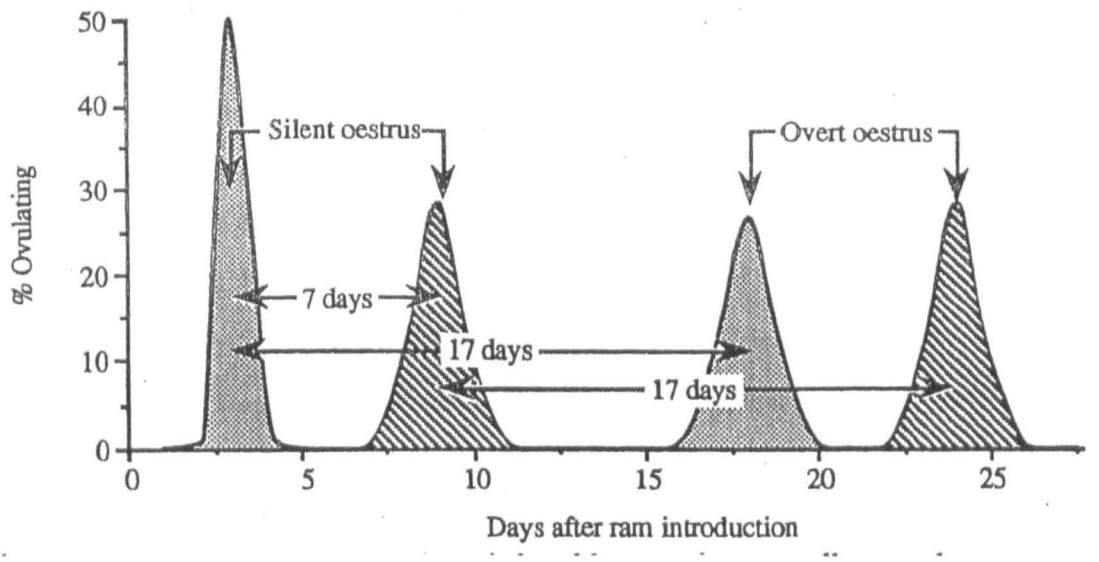


Figure 2.1 The patterns of ovulation and oestrus induced by introduction of rams to anoestrus ewes. Oldham and Martin, (1978)

2.1.3 MODE OF ACTION OF THE RAM EFFECT

Discovery of the ram effect led to investigations into how the ram stimulus can overcome the refractory state of the ewe during anoestrus and elicit an almost instantaneous increase in LH pulse frequency. The hypothesis behind the mode of action of the ram affect has been modified over the years (Review; Martin et al., 1986). Two main mechanisms have been proposed, a reversal of the negative effect of oestradiol at the level of the hypothalamus (Martin and Scaramuzzi, 1983) and alternatively a direct effect on LH release that is independent of oestradiol (Martin *et al.*, 1980). Martin *et al.*, (1983c) implanted ovariectomised ewes with oestradiol implants in spring (anoestrus) and oestrogen and progestagen implants during autumn (breeding season) to mimic the reduction in LH pulse frequency observed during both anoestrus and the luteal phase. Ram introduction to these modified ewes resulted in a rapid increase in LH pulse frequency (Martin *et al.*, 1983c). However even in the absence of steroid implants, ram introduction was associated with an increase in the basal levels of LH in ovariectomised ewes (Martin *et al.*, 1983c). Martin *et al.*, (1986) concluded a direct effect of the socio-sexual cues from the ram that bypasses the neural circuitry that constrains LH release. They suggested that it was merely easier to detect this effect in ewes under the influence of seasonal suppression of LH by oestradiol due to the low pulse frequency prior to ram introduction (Martin *et al.*, 1986).

2.1.3.1 PHEROMONES

Pheromones play a critical role in male and female communication in rodents (Review; Izard, 1983) thus it was deduced that they would play a critical role in male to female communication in sheep (Martin *et al.*, 1986). There are two types of pheromones, specifically primer pheromones that elicit a physiological response and releaser pheromones that modify an animal's behaviour (Reviews; Silverman 1977; Brennan and Keverne, 2004). The chemical substance or pheromone associated with elicitation of an LH response in anoestrous ewes exposed to a ram is released from sebaceous glands in the skin and wax collected from the ante orbital region of the eye (Knight and Lynch, 1980). Ram urine is not effective in stimulating an endocrine response in anoestrous ewes (Knight and Lynch, 1980) in contrast to evidence in rodents (Review; Izard, 1983). The capacity for ewes to respond to rams wool alone indicated that the endocrine response observed when rams are introduced to

anoestrous females was due to a specific primer pheromone similar to that in rodents. Furthermore wether lambs treated with testosterone are as effective as rams in inducing ewes to ovulate by the ram effect (Fulkerson *et al.,* 1981).

2.1.3.2 NON-OLFACTORY STIMULI

The mediation of the ram effect by olfactory stimuli is well documented in the literature (Knight and Lynch, 1980; Rekwot *et al.,* 2001; Signoret *et al.,* 1982). However more recent work emphasises the concept of a synergistic relationship between pheromones and the other exteroreceptive cues of behavioural, audio, visual and tactile stimuli (Cohen-Tannoudji *et al,* 1986; Pearce and Oldham, 1988; Perkins and Fitzgerald, 1994). Consideration of the ram stimulus as a combination of socio-sexual cues is an important concept when considering the ram effect, however there is some division in the literature as to importance of the non-olfactory stimuli. Morgan *et al,* (1972) found that ewes with an impaired sense of smell did not exhibit oestrus in response to ram introduction thus inferring that non-olfactory cues are insufficient, in the absence of chemical stimulation, to elicit an ovulatory response to the ram effect. However, in contrast Cohen-Tannoudji *et al.,* (1986) found that anosmic ewes (identified as anosmic using a repulsion test and post-experimental dissection) responded to ram exposure with a comparable LH response to intact ewes. Furthermore exposure of the same sample of ewes to wool extracts alone resulted in a zero response rate. This theory is further supported by the work of Pearce and Oldham, (1988) that examined the effects of restricting certain aspects of the ram stimulus on the ovulatory response of adult ewes. Specifically ewes were given full ram contact, opaque or clear fence line ram contact or isolated from rams prior to mating. Pearce and Oldham (1988) found a progressive increase in the number of ewes ovulating from no contact to visual contact through to a maximum ovulatory response from full ram contact. Therefore it was concluded that though pheromonal involvement undoubtedly forms a functional part of the ram stimulus, the ram effect is not associated solely with olfactory cues and that the other socio-sexual cues are of critical importance to the expression of this phenomenon (Pearce and Oldham, 1988).

Identification of the ram as a mate is important to coordination of male and female activity (Review; Pfaus *et al.,* 2001). Kendrick and Baldwin (1987) identified the ability for sheep to distinguish between visual characteristics of other sheep and

animals that thus permits differentiation between sexes, dominance ranking and the potentially threatening faces of dogs and humans. Of particular interest is the preference of anoestrous females for female faces and a change in preference towards male faces when the same ewes were in oestrus (Kendrick *et al.,* 1995). This evidence shows that sheep are able to distinguish between faces of individuals within and between breeds and sexes (Kendrick *et al.,* 1995). The shift in gender preference with physiological state also supports evidence of ewes actively seeking rams during the oestrous period (Fletcher and Lindsay, 1968).

2.1.3.3 DETECTION AND PROCESSING OF THE SOCIO-SEXUAL CUES

In mammals olfactory signals are detected through one of two pathways; the accessory olfactory system and the main olfactory system. Each of these systems is physiologically separate from the other both in terms of anatomical location and function. Figure 2.2 outlines the current knowledge regarding the role and anatomical pathways of these two systems in rodents and in sheep.

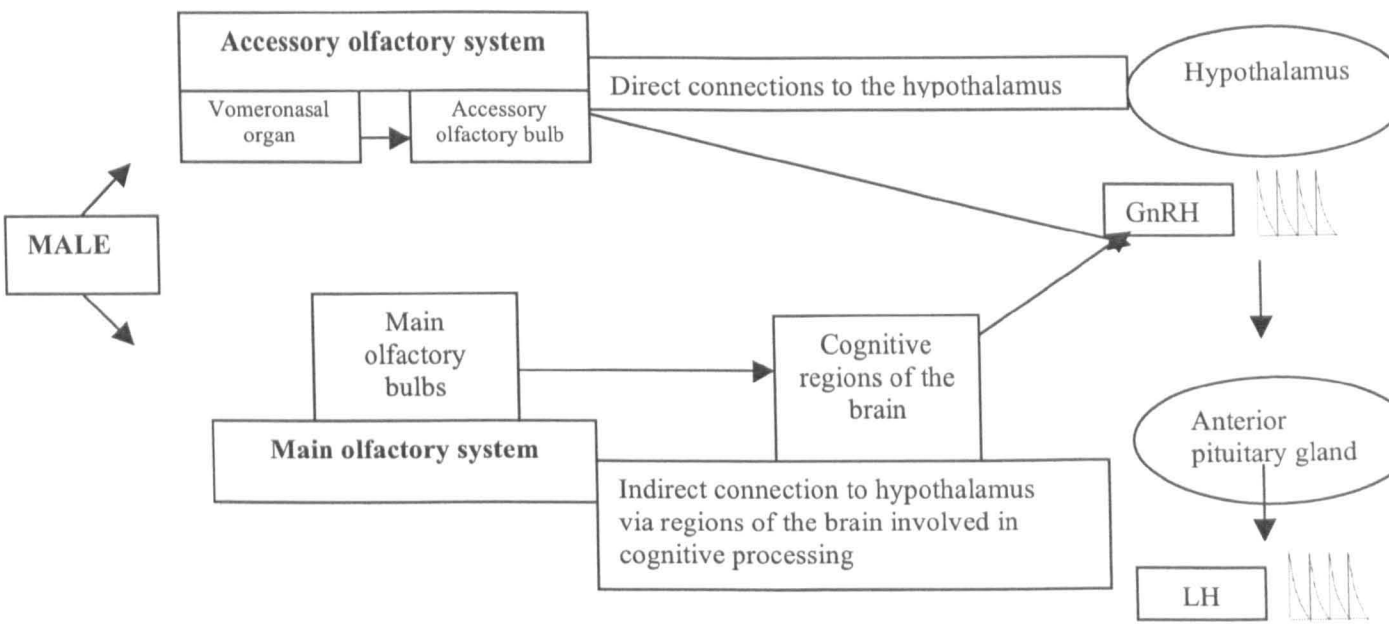


Fig 2.2 Schematic representation of the roles of the accessory and main olfactory systems in male elicited endocrine and behavioural responses in rodents and sheep.

1. **Accessory olfactory system** – The vomeronasal organ (VNO) and the accessory olfactory bulbs (AOS).

Chemosignals are detected by receptors in the vomeronasal organ that has axons projecting into the accessory olfactory bulb. This message is then communicated directly to the hypothalamus due to projections from the accessory olfactory bulb into the ventromedial and preoptic area of the hypothalamus (rat; Scalia and Winans, 1975, sheep; Jansen *et al.*, 1998).

Rodents – Critical role in detection of chemosignals that induce a neuroendocrine response; Example; male induced LH response and spontaneous abortion on exposure to a novel male (Bruce, 1960). Destruction of the VNO blocks the endocrine response to the chemosignals responsible for these processes (Wysocki and Lepri, 1991)

Sheep - Destruction of the VNO does not restrict the LH response to ram introduction in sexually experienced ewes (Cohen-Tannoudji *et al.*, 1989). Destruction of the main olfactory epithelium whilst leaving the accessory olfactory system intact renders ewes incapable of responding with an LH response to ram introduction (Gelez and Fabre-Nys, 2004). However introduction of a ram to ewes induces a significant increase in Fos activation in the both the accessory and main olfactory bulbs (Gelez et al., 2002) thus indicating an as yet undetermined accessory role in the detection and processing of ram odour (Gelez and Fabre-Nys, 2004)

2. Main olfactory system.

Chemosignals mediated through the main olfactory system (MOS) are subject to a degree of neural processing within the brain before acting upon the hypothalamus due to projections from the main olfactory system into the processing regions of the brain (Jansen *et al.*, 1998).

Rodents - Introduction of a sexual partner does not induce an increase in Fos activation in the main olfactory bulb (Halem *et al.*, 1999). Destruction of the olfactory epithelium does not modulate the responses to male urine or pregnancy block by novel male (Review, Gelez and Fabre-Nys, 2004). Furthermore the main olfactory system cannot compensate for the destruction of the vomeronasal organ (Wysocki and Lepri, 1991) thus indicating that

pheromonal cues from the male are mediated exclusively through the accessory olfactory system (Gelez and Fabre-Nys, 2004).

Sheep - As outlined above the main olfactory system is crucial to the mediation of the ram effect by the ram pheromone alone (Gelez and Fabre-Nys, 2004). However when the olfactory epithelium is obliterated completely and ewes are incapable of smell (anosmia), sexually experienced ewes exposed respond to a ram with an endocrine response that is comparable to intact ewes. Anosmic ewes exposed to rams fleece alone do not exhibit an LH response (Cohen-Tannoudji *et al.,* 1986)

2.2 FACTORS AFFECTING THE RESPONSE OF EWES TO THE RAM EFFECT

2.2.1 DEPTH OF ANOESTRUS

Depth of anoestrus both between and within breeds is critical to the predictability of successful ovulations in response to the ram effect (Lindsay and Signoret, 1980). Less seasonal breeds of sheep such as the Merino have a significantly greater propensity to ovulate in response to the ram effect with an estimated ovulatory response rate of between 40 and 100% (Martin, 1984). In contrast however, the efficacy of the ram effect is considerably more limited in more seasonal breeds of sheep such as Suffolk and Scottish Blackface ewes resulting in limited application of the ram effect in these breeds (Knight, 1980). However it is interesting that the reduced ovulatory response of Suffolk ewes during anoestrus does not appear to be due to an inability to acutely release LH in response to ram introduction (Minton *et al.,* 1991). The depth of anoestrus is a function of endogenous breed seasonality and climate, thus Merino ewes in the more temperate UK climate would not respond as readily to the ram effect as Merinos kept in more tropical climates.

A widely used measure of depth of seasonality both between breeds and over time during the annual cycle of reproductive events is the numbers of ewes spontaneously ovulating. Lindsay and Signoret (1980) demonstrated clearly that the percentage of ewes ovulating in response to the ram effect was directly proportional to the percentage of ewes spontaneously ovulating at the same stage of the annual reproductive cycle. Pearce and Oldham (1984) however also suggested that this might

be a function of the spontaneously cycling ewes facilitating the response of the anovular ewes to the ram introduction. This theory is widely supported by the extensive evidence of a positive effect of exposure of rams to oestrous ewes (Knight, 1985; Knight *et al.,* 1998) and introduction of rams with oestrous ewes (Nugent and Notter, 1990) on their ability to induce successful ovulations in anovular ewes. However this topic will be discussed fully in Chapter 2.3

The proportion of ewes that return to an anoestrous state after ovulating in response to the ram effect is heavily dependent on the timing of the initial ram introduction relative to the onset of the natural breeding season (Oldham and Cognie, 1980). There is a direct relationship between the proximity of the breeding season and the number of ewes having sustained oestrous cycles. This is of particular relevance in the more seasonal breeds of sheep that typically only have an acceptable level of ovulatory response to ram introduction towards the end of the anoestrous period (Rosa and Bryant, 2002)

2.2.2 DURATION OF RAM PRESENCE

Several studies have shown that maintained ram presence is necessary to maintain the occurrence of oestrous cycles within ewes induced to ovulate by the ram effect (Signoret *et al.,* 1982; Oldham and Pearce, 1983; Murtagh *et al.,* 1984b). Murtagh *et al.,* (1984b) found that continued ram presence enhanced the proportion of ewes exhibiting regular oestrous cycles after ram introduction. The work of Oldham and Pearce (1983) clearly showed that removal of the rams from anoestrous ewes after 6 hours was associated with a return of LH pulse frequency to pre ram exposure levels. Furthermore Signoret *et al.,* (1982) identified a direct relationship between the duration of ram presence and the percentage of anovular ewes ovulating in response to the ram effect.

2.2.3 ISOLATION AND THE ISSUE OF NOVELTY

The requirement for isolation of anoestrus ewes from within 500m metres of rams prior to ram exposure was first identified by Underwood *et al.,* (1944) and has been utilised as a template for conventional ram effect studies undertaken during anoestrus (Martin *et al.,* 1985; Perkins and Fitzgerald, 1994). However work undertaken by Oldham and Cognie (1980) demonstrated that two weeks was a sufficient isolation

period for a full ovulatory response to the ram effect. Furthermore Cohen-Tannoudji and Signoret (1987) demonstrated that a period as short as 24 hours was sufficient isolation for elicitation of an LH response during subsequent short term (3 hour) ram exposures, however the endocrine response was not followed through to ovulation.

As previously outlined, continued ram presence is thought to be necessary for sustained endocrine and ovulatory responses to the ram effect (Pearce and Oldham, 1983). However Oldham and Martin, (1978) found a decline in reproductive function and a return to an anoestrous state even in the continued presence of rams and concluded a possible development of resistance or reduced sensitivity to the ram stimulus. These observations are supported by the work of Riches and Watson (1954) where Merino ewes maintained in continuous contact with rams had a similar level and distribution of reproductive activity to that of ewes isolated from ram contact. These observations were deduced to be due to the ewes becoming habituated to the presence of the rams and is one of the key factors driving the perceived prerequisite of isolation of the sexes for effective application of the ram effect (Walkden-Brown *et al.*, 1999).

This concept of habituation to the sexual stimulus is well represented in the literature (Review; Dewsbury, 1981). However evidence from studies in rodents (Coquelin and Bronson, 1971) have identified that this type of sexual habituation can be overridden by the introduction of a novel female, a theory termed the 'Coolidge effect'. This is by definition the resumption of copulatory ability of a sexually satiated individual by the introduction of a novel animal and occurs across species (Review; Dewsbury, 1981). Relative to sheep, Pepelko and Clegg, (1965) found that rams show a depletion in sexual behaviour during 20 minutes of exposure to an oestrous female that can be re-stimulated by introduction of a novel female. Furthermore Cushwa *et al.*, (1992) found that a high proportion of anovular ewes maintained in continuous contact with rams ovulated in response to the introduction of a novel ram.

Gelez *et al.*, (2004) proposed that the complex nature of the ram pheromone might permit individual coding to allow olfactory discrimination between rams. The capacity for olfactory learning in sheep is evident in the ability of the ewe to distinguish between her own and alien offspring within 4 hours of birth (Levy *et al.*,

1995). This process of pheromonal learning is mediated through the main olfactory system (Levy *et al.*, 1995), which is also the primary route for mediation of sociosexual cues in the ewe (Gelez et al., 2004c). Furthermore recent evidence of the capability of sheep to discriminate between males and females (Kendrick *et al.*, 1995) and between familiar and unfamiliar individuals (Piece *et al.*, 2000) using visual stimuli alone supports the possible capability of ewes being able to distinguish between individual rams.

2.2.4 POTENCY OF THE RAM STIMULUS

The quality, type and duration of ram stimulus are critical to extent of ovulatory response to the ram effect (Walkden-Brown *et al.*, 1999). The effectiveness of androgen treated wethers and females in stimulating ovulation in anovular females (Fulkerson *et al.*, 1981) is compelling evidence that pheromone production is a strong determinant of the capability of a ram to induce ovulation in anovular females. Therefore it is logical that any factors affecting the circulating levels of testosterone will affect the quality and thus efficacy of the ram stimulus (Perkins and Fitzgerald, 1994).

The importance of behavioural stimuli is highlighted by the work of Perkins and Fitzgerald (1994) where the proportion of ewes ovulating was significantly greater within ewes exposed to rams of a higher sexual libido. These rams performed more courtship activity thus inferring the importance of behavioural, tactile, audio and visual cues to successful induction of the ram effect. This theory is supported by the increased efficacy of androgen treated wethers in inducing ovulation when selected for high compared to low libido (Fulkerson *et al.*, 1981).

Endogenous levels of testosterone vary between the breeding and non-breeding season due to the seasonal suppression of LH that is directly related to testosterone production and spermatogenesis (Gonzalez *et al.*, 1989). This annual variation thus affects the potency of the ram stimulus and can have connotations on application of the ram effect to out of season breeding. This is of particular significance in very seasonal breeds, where the ewe has an innate lower propensity to ovulate to the ram effect (Oldham and Cognie, 1980; Minton, 1989). This in conjunction with a reduced

potency of the ram contributes to the poor reproductive response to the ram effect in seasonal breeds of sheep.

2.2.5 PREVIOUS SEXUAL EXPERIENCE

Beach (1947) identified the importance of sexual experience early in reproductive development on the ability of an animal to respond to subsequent sexual encounters with the appropriate behavioural and endocrine responses. Sexual experience is critical to the development of a rapid opportunistic endocrine and behavioural responses that will result in optimal efficiency and efficacy of mating (Graham and Desjardins, 1980; Woodson, 2002). Pfaus *et al.*, (2001) proposed that a mate is an amalgamation of stimuli that may or may not inherently elicit a sexual response in an individual of the opposite sex. They proposed that with sexual experience, stimuli that were initially ineffective at inducing a sexual response become associated with the anticipation and expectation of sexual rewards.

2.2.5.1 THE EWE

Maiden (sexually naïve) ewes typically show a poorer level of reproductive competence than adult ewes and this is true for ewes bred as ewe lambs (5-12 months) up to those bred as yearlings (18 months). Rosciszewska (1985) identified a reduced receptivity of the maiden ewes to the rams compared to adult ewes that thus increased the proportion of mounts per ejaculation and failed mounts per mating. Gelez *et al.*, (2004a) identified that sexually naïve maiden ewes were both less receptive and proceptive to the ram.

Recent work has shown that the interplay between dopamine and noradrenalin in the medial basal hypothalamus (outlined in Chapter 1.1.3.1) is not present in sexually naïve ewes (Gelez *et al.*, 2004a). The hypothesis proposed by Gelez *et al.*, (2004a) revolves around a key role of experience in the development of this pattern of neurotransmitter release in the medial basal hypothalamus. Mesolimbic dopamine is important in reward and motivational mechanisms (Robbins and Everitt, 1996) and levels of dopamine have been shown to increase in rodent females during intromission but with this elevation amplified in sexually experienced animals (Kohlert *et al.*, 1997). Gelez *et al.*, (2004a) therefore proposed that experience is vital in the

association of the socio-sexual cues of the male with mating activity. They suggested that the neural reward received in response to intromission increases over a number of sexual interactions and that this is critical in the development of the receptivity in the sexually naïve ewe.

Little work to date has focused on the role of sexual experience on the responses of the ewe to the ram effect. Murtagh *et al.,* (1984a) identified a greater ovulatory response in Merino ewe lambs exposed to the ram at 12 months when subsequently re-introduced to the ram at 15 months of age thus supporting the possibility of augmentation of female performance as a consequence of experience. Gelez *et al.,* (2004c) found that ewes preconditioned with a two-week ram exposure period had a significantly greater increase in LH pulse frequency than sexually naïve maiden ewes when exposed to rams fleece. In contrast, they found no significant difference between the endocrine responses of sexually naive and sexually experienced maiden ewes when introduced to the entire ram (Gelez *et al.,* 2004c). However these observations were confounded by the exposure of the same maiden ewes to rams fleece two days prior to ram introduction. Therefore it is impossible to conclude whether the naïve maiden ewes responded irrespective or because of this prior exposure to the rams fleece.

2.3 THE FEMALE EFFECT

The physiological effect of exposure to socio-sexual cues is not restricted to an effect of the ram on the ewe; indeed exposure of ewes to rams stimulates an increase in LH pulse frequency in the rams (Sanford *et al.,* 1974; Gonzalez *et al.,* 1988). This endocrine response is not dependent on the ewe being sexually receptive, however it is enhanced if the ewe is in oestrus (Gonzalez *et al.,* 1991a). All potential sources of a 'female pheromone' have been investigated by exposure of rams to masks containing female fleece, urine and vaginal secretions (Gonzalez *et al.,* 1991b). However in contrast to the investigation into the source of the 'ram pheromone' (Knight and Lynch, 1980) there was no endocrine response within rams exposed to the female odours. Signoret (1991) proposed an alternate origin of the female pheromone to those tested or that the proximity to the stimulus permitted by the masks was insufficient to elicit an endocrine response.

2.3.1 EFFECT OF EXPOSURE OF RAMS TO EWES

Exposure of rams to oestrous ewes prior to use for the ram effect stimulates an increase in LH and testosterone (Kridli and Said, 1999) in both sexually experienced and inexperienced rams (Gonzalez *et al.*, 1991a). In addition to the LH response to female introduction, rams have been reported to have an increase in cortisol when exposed to oestrous females (Gonzalez *et al.*, 1988). This response was dependent on direct ram-ewe contact and occurred concurrently with an increase in LH pulse frequency. Both responses were enhanced by mating and ejaculation (Gonzalez *et al.*, 1988).

This type of priming is typically also associated with an increased expression of sexual behaviour (Rosa *et al.*, 2000), the importance of which has been outlined in Chapter 2.2.4. However there is some conflict in terms of the practical implications of this female effect as Rosa *et al.*, (2000) found that although exposure to oestrous ewes caused an increase in ram sexual behaviour this was not necessarily associated with an improved ability to induce ovulation in anovular ewes.

2.3.2 EFFECT OF EXPOSURE OF EWES TO OESTROUS EWES

The effectiveness of stimulation of anoestrous ewes with oestrous ewes remains a contentious issue in the literature. Knight (1985) identified no significant stimulatory effect of the introduction oestrous ewes to anoestrous ewes. This is supported by the observations of O'Callaghan *et al.*, (1994) where there was no significant effect of oestrous ewes on LH pulse frequency in anoestrous ewes maintained under natural photoperiod. Furthermore Yildiz *et al.*, (2002) identified a suppression of LH pulse frequency in anoestrous ewes exposed to oestrous females. However O'Callaghan *et al.*, (1994) observed that ewes maintained in a group had a significantly earlier onset of reproductive activity than ewes maintained in isolation under an artificially maintained photoperiod and proposed a greater role of ewe-to-ewe interactions in the absence of a changing photoperiod.

In contrast Zarco *et al.*, (1995) provided compelling evidence of a direct effect of contact with oestrous ewes on the proportion of anoestrous ewes with luteal activity. However their evidence is confounded by the use of intermittent ram exposures to detect oestrus. Although rams were used to detect oestrus in ewes maintained at

varying levels of proximity with oestrous ewes, it cannot be discounted that the proportion of oestrous ewes and the exposure of the rams to these ewes may have affected the observed result. The effect of oestrous ewes on the behaviour and stimulus quality of rams is widely accepted in the literature (Knight, 1985, Nugent and Notter, 1990), hence it cannot be discounted that this study may simply be a further demonstration of the effect of oestrous ewes on the efficacy of the ram in stimulating anoestrous ewes.

2.4. OTHER APPLICATIONS OF THE RAM EFFECT

2.4.1 ADVANCEMENT OF PUBERTY

The prominent effects of photoperiod and physiological age can confound the evidence of an effect of the ram on the onset of puberty (Martin, 1984). Under normal conditions, maiden ewes must be exposed to a specific photoperiodic regime prior to attainment of puberty specifically long days associated with summer, followed by short days of approaching autumn and winter (Yellon and Foster, 1985). This can be overcome by exposure to rams (Knights *et al.,* 2002) though the evidence is not equivocal. Al-Mauly *et al.,* (1991) found that though ram introduction to spring born ewe lambs during August and September stimulated an increase in basal LH (August) and LH pulse frequency (September) neither were sufficient to immediately induce ovulation in the ewe lambs. Only ram introduction during October was directly responsible for induction of ovulation. However in all ram-exposed ewes, the mean date of the first ovulation occurred significantly earlier than ewes maintained in isolation from ram contact. This infers that the ovulation was not solely an effect of the advancing photoperiod and older physiological age and that the ram effect could only be directly attributed to the attainment of puberty in combination with one or both of the above factors. This is in agreement with observations in autumn born lambs where ram introduction during late July but not early July or May directly stimulated the first ovulation (Lopez- Sebastian *et al.,* 1985 cited by Knights *et al.,* 2002).

2.4.2 MANIPULATION OF REPRODUCTION DURING THE BREEDING SEASON

Very few previous studies have investigated the endocrine responses of cyclic ewes to ram introduction. The introduction of rams to ewes during the breeding season is

associated with a reduction in the length of the follicular phase and an advanced LH surge and ovulation relative to luteolysis (Lindsay *et al.*, 1975). Pearce and Oldham, (1983) found that ovariectomised ewes implanted with oestradiol and progesterone responded to ram introduction during the breeding season with an increase in both basal and pulsatile LH. Furthermore ovariectomised ewes implanted with oestradiol only had an increase in basal LH in response to ram introduction (Pearce and Oldham, 1983). Ngere and Dzakuma, (1975) found that introduction of rams to randomly cycling tropical ewes resulted in a large number of ewes mated within the first day of ram introduction, however there was no clear indication of the mechanism that caused this skewed distribution of mating.

2.5 SOCIO-SEXUAL INTERACTIONS IN OTHER SPECIES – SIMILARITIES AND DIFFERENCES WITH OBSERVATIONS IN SHEEP

2.5.1 RODENTS

Male and female rodents respond to introduction of the opposite sex with an increase in LH pulse frequency similar to that observed in sheep. Stimuli responsible for both the male and female effect in rodents are mediated through the accessory olfactory system (Whitten, 1959) as outlined in Chapter 2.2.1.3.

In contrast to sheep, the concept of female-to-female oestrus synchrony is a well-established principle in rodents (McClintock, 1978). Identification of the stage of oestrous cycle with vaginal smears permits analysis of direct female-to-female synchrony in the absence of the confounding effect of the male (Mclintock, 1978). Mclintock (1978) proposed that synchrony of 50-75% developed over a minimum of three oestrous cycles by the lengthening and shortening of oestrous cycles. The capacity of re-circulated air supply to induce a similar degree of synchrony indicates that the stimulus is air borne and chemical in nature (Mclintock, 1978). In contrast to sheep, the efficacy of the male effect is attenuated if rats are not maintained in groups. This is due to the above entrainment of oestrous cycles within group-housed rats that thus permits a synchronous response to introduction of the male (Whitten, 1959).

In contrast to sheep, the role of novelty in the stimulation of endocrine responses is well documented in rodents. Male mice exposed continuously or repeatedly to the

same female show a gradual depletion in the LH response to introduction of the same female (Coquelin and Bronson, 1979). However the introduction of a novel female resulted in an LH response comparable to that stimulated by the first exposure to the initial female (Coquelin and Bronson, 1979). The capacity of rodents to distinguish between pheromones from a familiar and novel individual is further evident in the 'Bruce effect'. Exposure of pregnant mice to a novel male induces abortion due to detection, identification and elicitation of a specific physiological response to the novel odour (Bruce, 1960).

The first observation of male induced puberty was in mice. Vandenburgh (1967) observed that rearing of female mice in the presence of adult males advanced the attainment of puberty. They identified a pheromone in the male urine responsible for inducing the cascade of events leading to the first preovulatory LH surge and ovulation (Vandenburgh, 1967). The absence of seasonality in mice is in marked contrast to sheep and thus makes identification of male induced puberty more easily identifiable.

Graham and Desjardins (1980) demonstrated the concept of olfactory learning associated with sexual encounters in rats through a Pavlovian style association between environmental cues and the sexual encounter. The male was exposed to a conditioning stimulus (methyl salicylate) and then to a sexually receptive female. After a conditioning period, exposure of the male to the conditioning stimulus alone resulted in a significant elevation in levels of LH similar in magnitude to exposure of the male to a sexually receptive female. The ability of the male to respond with a characteristic LH response to certain environmental cues previously associated with a female demonstrates the capacity for learning and association with this type of sexual response (Graham and Desjardins 1980).

2.5.2 GOATS

Introduction of bucks to anoestrous does induces an acute increase in LH pulse frequency similar to that observed in the ewe (Shelton, 1960; Chemineau, 1987). As with the ram, exposure of the doe to the full complement of socio-sexual cues associated with the buck increases the ovulatory response to the buck effect (Chemineau, 1987; Walkden-Brown *et al.*, 1993). Furthermore abolition of the sense

of smell in adult sexually experienced goats does not modify the LH secretion to introduction of the buck (Chemineau *et al.,* 1986).

Within goats a female effect is firmly established with compelling evidence in the literature of stimulation of anoestrous does with artificially induced cyclic goats (Walkden-Brown et al., 1993, Restall *et al.,* 1995). Introduction of the buck to cyclic goats appears to affect the distribution of oestrous cycles with a higher than expected proportion of does mated on Days 1 and 2 after buck introduction (Chemineau, 1983). This is similar to the observations in cyclic ewes where the frequency of mating on Day 1 after ram introduction was higher than that expected by chance (Ngere and Dzakuma, 1975). Chemineau (1983) proposed a possible luteolytic effect of the buck, however as in the study in cyclic ewes (Ngere and Dzakuma, 1975) there is no conclusive evidence as to how this 'buck effect' is mediated during the breeding season.

2.5.3 PIGS

Boar contact is commonly used in commercial pig production to advance puberty in pre-pubertal gilts (Reviews, Hughes *et al.,* 1990; Evans and O'Doherty 2001b). Age at first exposure is critical to the efficacy of the effect with an optimal age at first exposure of 165 days (Peacock and Hughes, 1995). In association with a boar-induced increase in LH pulse frequency, pre-pubertal gilts also undergo a parallel increase in concentrations of cortisol (Pearce and Hughes, 1987; Kingsbury and Rawlings, 1993). Pearce *et al.,* (1988) investigated the effect of acute elevations in plasma cortisol on responses to exogenous GnRH administration and endogenous LH release. The LH response to exogenous GnRH administration was reduced when accompanied by a concurrent cortisol challenge. However in the absence of administration of exogenous GnRH, the cortisol challenge increased rather than suppressed LH secretion in prepubertal gilts. Pearce *et al.,* (1988) proposed that the acute elevation in cortisol in response to full boar contact may be involved in mediating changes to the hypothalamic-hypophyseal axis to steroid feedback (Pearce *et al.,* 1988). However in later work, Pearce and Patterson (1992) concluded that full boar contact was most effective in stimulating puberty in prepubertal gilts because it allowed optimal physical contact between the boar and the gilt rather than because it elicited a cortisol response. The specific role of cortisol in boar-induced stimulation of puberty in gilts remains undetermined.

Turner *et al.*, (1998) found that post pubertal gilts introduced to boars during both the luteal and follicular phases of their oestrous cycle have a significant increase in cortisol in response to boar introduction. Gilts experiencing entire boar contact (but not fence line contact) had shorter oestrous cycles and a higher ovulation rate and the authors implied a possible positive effect of this type of boar-induced stress (Turner *et al.*, 1988).

There is strong evidence in the literature of a female effect in the stimulation of puberty in gilts (Pearce, 1992; Pearce and Pearce, 1992). However it appears to be driven by the female being a strange individual rather than specifically associated with the oestrus or anoestrous state of the female (Peacock and Hughes, 1995).

2.5.4 CATTLE

The presence of a bull can advance the onset of puberty in pre-pubertal heifers (Izard and Vandenburgh, 1982) and advance the resumption of cyclic activity post partum (Izard, 1983). However the evidence in cattle is not equivocal and success of biostimulation can be unpredictable and unreliable (Review, Rekwot *et al.*, 2001). Possibly due to the predisposition for a negative energy balance in post partum cattle, nutrition plays a critical role in determining the physiological responses of cows to the bull (Monje *et al.*, 1992). There is some evidence of female-female stimulation in reducing the period of post partum anoestrus however an effect is only evident in cattle predisposed to an extended anoestrous period (Wright *et al.*, 1994).

2.5.5 HUMANS

As in rodents, the concept of female-to-female induced menstrual synchrony has been widely investigated in the literature. Since the initial findings of McClintock (1971) it is accepted that in humans, menstrual synchrony can develop within family groups, roommates and close friends (Review; Weller and Weller, 1993). Mclintock (1971) proposed a similar model to that outlined above for rodents of a lengthening and shortening of menstrual cycle length driven by chemical communication. It has been proposed that proximity is not necessarily sufficient to synchronise menstrual activity and that individuals need to interact on both a physical and social level (McClintock, 1971; Quadagno *et al.*, 1981). However the evidence is far from equivocal and is

further complicated by disagreement in the methods of measurement of synchrony (Wilson, 1992; Schank, 2000, 2001) and natural variation in cycle length (Weller and Weller, 1997).

Evidence of a male effect in humans is lacking in the literature. McClintock, (1971) reported some variance in individual menstrual cycle length that was dependent on the presence of a male partner. However Jarett (1984) and Quadagno *et al.*, (1981) found that the presence of a male partner was not a reliable predictor of menstrual cycle length or cycle regularity. Quadagno *et al.*, (1981) proposed that perpetual association with males in daily life might obscure any effects that male exposure may have on female reproductive activity in humans.

2.6. INCORPORATION OF THE RAM EFFECT WITH REPRODUCTIVE TECHNOLOGIES

2.6.1 MELATONIN

Changing day length is coded in the sheep by the pattern of melatonin secretion that thus stimulates changes in the sensitivity of the hypothalamic-hypophyseal axis to the negative effects of oestradiol (Karsch *et al.*, 1984). Treatment with melatonin can advance the onset of the breeding season and improve reproductive performance (Gomez Brunet *et al.*, 1995). As mentioned previously, the depth of anoestrus is a major factor in determining the ovulatory response of ewes to the ram effect (Lindsay and Signoret, 1980). Treatment with melatonin can effectively alter the seasonal endocrine state of the ewe and in combination with melatonin implants markedly improve the ovulatory response of anoestrous ewes to the ram effect (Zuniga *et al.*, 2002).

2.6.2 ARTIFICIAL SYNCHRONISATION PROTOCOLS

The majority of work investigating the interaction between exposure to rams and controlled breeding programmes has focused on the influence of the ram after removal of the influence of the artificial progestagen (Lewis *et al.*, 1974) and has been undertaken predominantly during anoestrus (Romano *et al.*, 2001). Several studies identified acceleration of oestrous onset and shortening of the duration of oestrus as a result of continuous ram presence post sponge removal (Maxwell, 1986; Romano *et al.*, 2000; 2001). These observations are in accordance with work undertaken in

anoestrous ewes in the absence of progestagen where continuous presence of the ram compared to intermittent ram presence shortened oestrous onset and duration (Parsons and Hunter, 1967; Fletcher and Lindsay, 1971).

2.6.3 ARTIFICIAL INSEMINATION.

The ram effect can be used as the method of oestrus synchronisation in anoestrous ewes prior to artificial insemination (Corke, 1980) or after progestagen synchronisation to influence the time of ovulation relative to progestagen withdrawal (Lucidi *et al.,* 2001). Lucidi *et al.,* (2001) found a significantly higher pregnancy rate in ewes exposed to rams after withdrawal of the artificial progestagen. However this may be merely a reflection of ram-induced enhancement of synchrony within the treated ewes and accurate timing of insemination relative to ovulation. Lucidi *et al.,* (2001) found that ram exposure post progestagen withdrawal advanced the LH surge and ovulation and thus inseminated the ewes earlier (50 hours after progestagen withdrawal) to compensate for this. In a commercial situation an unexpected alteration in the timing of endocrine events post progestagen withdrawal may have resulted in lower conception rates due to asynchrony between ovulation and insemination.

The ram effect is used in Australia as a low input method of non-pharmacological oestrus synchronisation for artificial insemination of ewes during anoestrus (Corke, 1980). Conception rates to artificial insemination with both frozen and fresh semen are typically enhanced by insemination to a natural oestrus (Oleson, 1993). Therefore the potential of the ram as a non-pharmacological alternative method of oestrus synchronisation may enhance conception rates and litter size using frozen semen by allowing insemination to a synchronised natural oestrus.

2.7 THE APPLICATION OF SOCIO-SEXUAL INTERACTIONS TO UK SHEEP REPRODUCTION

The current knowledge of the manipulation of reproduction using socio-sexual cues is predominantly restricted to the anoestrous period. The potential for conventional application of the ram effect in the UK is restricted by the seasonality of many commercial breeds of sheep. Therefore I propose to investigate the potential for development of strategies using socio-sexual cues for manipulation and improvement of reproductive performance that are applicable to more seasonal breeds of sheep.

The following experimental chapters have three key aims:

1. The development of a non-pharmacological method of oestrus synchronisation using socio-sexual cues for natural mating and artificial insemination during the breeding season.
2. Investigation of the role of prior experience of the ram during the anoestrous period and the breeding season in modulating the subsequent endocrine and behavioural responses when introduced to rams during the transition between late anoestrus and the breeding season and during the breeding season.
3. Investigation of the potential benefits of combining artificial methods of reproductive control with the stimulation of ewes by socio-sexual cues.

3. EFFECT OF REPEATED FENCE LINE AND VASECTOMISED RAM EXPOSURE DURING THE TRANSITION INTO THE BREEDING SEASON ON THE SYNCHRONY OF MATING AND LAMBING

3.1 ABSTRACT

The ram effect is typically applied during anoestrus when the anovulatory state of the ewes permits a synchronous endocrine response to ram introduction. However in more seasonal breeds of sheep, predictable ovulatory responses to the ram effect are limited to the transition into the breeding season. In an attempt to synchronise mating later during the breeding season, mule ewes underwent 24 hour exposures to fenceline (Experiment 1; FR, n=94) or vasectomised ram contact (Experiment 2; VR, n=103) repeated at 17 day intervals (x3) from the transition into the natural breeding season. Control ewes (Experiment 1; FC, n=98 and Experiment 2; VC; n=106) remained isolated from ram contact prior to mating. A subset of VR (n=35) and VC ewes (n=35) were blood sampled twice weekly to monitor their pre-mating progesterone profiles. At mating, harnessed entire rams were introduced, 17 or 16 days after the last ram exposure (Experiments 1 and 2 respectively) and raddle marks were recorded daily. Repeated 24-hour exposure to both fenceline and vasectomised ram contact advanced the time ($P<0.001$) and compacted the distribution of mating (FR; $P<0.001$ and VR; $P<0.01$). At lambing ram-exposed ewes lambed earlier (FR; $P<0.01$and VR; $P<0.001$) with a more compact distribution of lambing ($P<0.01$). The progesterone data indicated that the earlier date of mating in the ram-exposed ewes was a result of an earlier onset of the breeding season ($P<0.05$) but that the synchrony of mating developed over the pre-mating period; a pattern which was not evident in the control ewes. Vasectomised ram exposed ewes tended to have a lower litter size than control ewes ($P<0.1$) in contrast to the fence line ram-exposed ewes where there was no difference. In conclusion, repeated, acute exposure of mule ewes to the ram during the transition into the breeding season is an effective method of altering the distribution of mating, when ewes are mated later in the breeding season.

3.2 INTRODUCTION

Oldham and Pearce (1984) identified the ram effect as a potential method of controlling sheep breeding to provide farmers with a cheap, reliable and non-pharmacological method of oestrus synchronisation. It is widely accepted that the ovulatory response to the ram effect decreases with the depth of anoestrus (Martin and Scaramuzzi, 1983). Therefore in spite of it's potential and widespread use in other climates (reviews: Martin *et al.*, 1986; Walkden-Brown *et al.*, 1999), widespread application of the ram effect to UK sheep production is limited by the deep seasonality of many British breeds of sheep (Al-Mauly *et al.*, 1991). Studies into the relevance of the ram effect to sheep production in the UK have focused mainly on conventional application to out of season breeding (Review: Rosa and Bryant, 2002) or on the advancement of puberty (Dyrmundsson and Lees, 1972; Al-Mauly *et al.*, 1991). These studies have mainly focused on incorporation with strategies to overcome the intrinsic seasonality of many British breeds of sheep such as with the use of melatonin implants (Rekik *et al.*, 1991; Donovan *et al.*, 1994, Rosa *et al.*, 2000) and following prior exposure of rams to oestrous ewes (Rosa *et al.*, 2000). However in the absence of exogenous melatonin the percentage of ewes ovulating in response to the ram only reached acceptable levels late in the anoestrous period (Al-Mauly *et al.*, 1991; Rosa *et al.*, 2000). Therefore it is likely that the greatest potential for response of seasonal breeds of sheep to the ram effect lies in closer proximity to the breeding season.

Studies into the application of the ram effect to the synchronisation of ewes during the breeding season have to date been limited to the incorporation into synchronisation procedures involving the use of exogenous hormones (Ungerfeld and Rubianes, 1999; Evans *et al.*, 2004; Hawken *et al.*, 2005). However such synchronisation procedures are unacceptable in organic production systems (Compendium of organic standards, 2004). Furthermore consumers are demanding more "clean, green and ethical" farm practices (Martin *et al.*, 2004). Therefore development of strategies for the predictable and reliable use of the ram effect would provide an alternative non-pharmacological method for oestrus synchronisation in the breeding season. The fluctuating sensitivity of the endocrine axis to the negative effects of oestradiol during the transition into the breeding season (Karsch *et al.*, 1984) thus presents a possible novel opportunity for ram-induced manipulation of cyclicity.

Within anoestrous ewes stimulated to ovulate by the ram effect, continued ram presence is necessary for the maintenance of oestrous cycles and to avoid ewes reverting to an anoestrous state (Oldham and Pearce, 1983). However Rosa and Bryant (2002) proposed that ewes exposed to rams during the transitional period, between anoestrus and the breeding season, maintained cyclicity until the onset of the subsequent anoestrous period. Furthermore, Martin *et al.*, (1986) found that two weeks was a sufficient period of isolation from the ram to permit a subsequent unaffected endocrine response to the ram. Therefore, I hypothesise that short duration ram exposures during the transitional period into the breeding season will induce an LH response and ovulation in a proportion of ewes. We propose that the removal, isolation and subsequent re-instatement of the ram may then induce an LH response in the remaining unaffected, non-cyclic ewes thus bringing their cycles in synch with their flock mates resulting in compacted and synchronous breeding and lambing.

These two experiments were designed to identify whether short duration (24 hour) exposures to a ram stimulus (fence-line or with vasectomised rams), repeated at intervals of approximately one complete oestrous cycle (17 days) from the transitional period into the breeding season will influence the timing and synchrony of mating. Based on evidence of the optimal affect of the ram effect when the ewe is exposed to the complete complement of male sensory cues, I hypothesised that the use of vasectomised rams would be more effective in inducing synchrony than fence line ram contact. I aimed to detect the impact of these types of periodic intermittent ram exposure on conception rates to first service and determine whether this strategy has potential as a non-pharmacological method of synchronisation of mating during the breeding season.

3.3 MATERIALS AND METHODS

3.3.1 EXPERIMENT 1

3.3.1.1 ANIMALS AND EXPERIMENTAL PROCEDURES

During September, 192 multiparous mule ewes (Scottish Blackface ewes crossed with Border or Blue-Faced Leicester rams) that had been previously isolated from ram contact (i.e. not maintained within 500m of any rams), were allocated to fence line ram-exposed (FR; n=94) and control groups (FC; n=98), balanced on age and parity and maintained at pasture at Cockle Park Farm, Northumberland (55°13'N). FR ewes underwent 24 hour fence-line contact with entire rams (Texel; n=7 and Suffolk; n=7) on Days 0 (September 10th), 17 and 34 of the experiment. The fence line contact was permitted by enclosure of the entire rams in a pen that was surrounded by 2m high perimeter netting to prevent direct physical contact between ewes and rams, located in a paddock (2000m^2). The entire rams were given access to hay and water, sufficient to sustain them over the 24-hour period. Between each 24-hour exposure period the FR ewes were isolated from ram contact and grazed on alternative pasture to the exposure paddock. FC ewes remained isolated from any contact with rams (entire or vasectomised) for the duration of the experimental period prior to mating.

The groups of FR and FC ewes were mixed on Day 51, 17 days after the last exposure of the FR ewes and raddled entire rams (n=10) were introduced for mating. Raddle marks were recorded daily to identify the timing and numbers of ewes mated during the first oestrous cycle and then recorded weekly for the subsequent 34 days. Raddle colour was changed on Days 14 and 32 after ram introduction to monitor ewes not conceiving to the first service. The ewes were maintained subject to conventional farm practice until lambing when the number of lambs and date of lambing was recorded.

3.3.2 EXPERIMENT 2

3.3.2.1 ANIMALS AND EXPERIMENTAL PROCEDURES

During August, multiparous mule ewes (previously isolated from ram contact) were assigned to either control (VC; n=106) or vasectomised ram-exposed groups (VR; n=103) based on age and parity and were maintained at pasture at Cockle Park Research Farm as detailed in Experiment 1. VR ewes were exposed to raddled, vasectomised rams (n=3) for 24 hours on Days 0 (September 10th), 17 and 34 of the

experiment. Between each 24 hour exposure period the VR ewes were isolated from ram contact. Raddle marks were recorded after each exposure with raddle colour changed before each exposure period. The vasectomised rams were rotated to ensure that one of the three vasectomised rams had never previously been exposed to the ewes to permit a degree of novelty; the other two vasectomised rams remained the same for all three exposures. VC ewes were isolated from any ram contact for the duration of the experimental procedure prior to mating as in Experiment 1.

VR and VC ewes were mixed on Day 50 of the experiment, sixteen days after the last vasectomised ram exposure of the VR ewes, and raddled entire rams (n=10) were introduced for mating. Raddle marks were recorded daily to identify the timing and numbers of ewes mated during the first oestrous cycle and then recorded weekly for the subsequent 34 days. Raddle colours were changed on Days 14 and 32 after ram introduction to permit identification of ewes not conceiving to the first service. Ewes were maintained subject to conventional farm practice until lambing, when the number of lambs and date of lambing were recorded.

3.3.3 BLOOD COLLECTION

Blood samples (5ml) were collected twice weekly by jugular venepuncture (Vacutainer, Becton-Dickinson Limited, Coventry) from a subset of VC (n=35) and VR ewes (n=35). Sampling commenced 2 weeks prior to the first vasectomised ram exposure on Day -13 (28th August) and continued until to Day 50 (30th October) to establish whether ewes were anovular prior to the V-ram exposure and to profile the progesterone secretion of VR and VC ewes during the pre-mating period. Blood samples were centrifuged as soon as possible (within 24 hours of collection) at 3000 rpm for 20 minutes. Plasma was decanted into duplicate plastic tubes (Sarstedt Ltd, Leicester, UK) that were capped, immediately frozen and stored at -20°C until analysis.

3.3.4 HORMONE ANALYSIS

Plasma progesterone concentrations were analysed in duplicate using a commercial enzyme linked immunoassay (ELISA) kit (Ridgeway Science Ltd, Gloucester, UK). 200µl of progesterone-enzyme label was added to 10µl of standard or sample and incubated for 2 hrs 20 minutes at room temperature. After three washes in buffer,

200µl of alkaline phosphatase substrate was added to each well and left for 20-30 minutes in the dark to incubate. After the 20-30 minute incubation period, the colour development of the standards and samples were compared at a primary wavelength of 570nm. Mean intra-assay and inter-assay coefficients of variation for low (2.26ng/ml), medium (3.52ng/ml) and high (7.13ng/ml) plasma samples were 6.9 and 11.7%, 5.1 and 11.2% and 6.9 and 8.8% respectively. The limit of sensitivity of the assay was 0.2ng/ml.

3.3.5 DATA ANALYSIS

3.3.5.1 BLOOD SAMPLED EWES ONLY

Blood sampled ewes (Experiment 2) were initially assessed for their anovular or cyclic status as for the purpose of this study we wanted to only consider ewes that were anovulatory at the time of the first ram exposure. A ewe was classified as cyclic prior to ram introduction if progesterone (during the four samples prior to the first ram exposure period) was elevated above 1.5ng/ml for at least 2 consecutive samples. All of the VR ewes were confirmed as anovular however one ewe had elevated progesterone above 1ng/ml for 13 samples indicating the presence of a persistent corpus luteum (CL) and was therefore excluded from data analysis. Within the VC ewes, 6 ewes were excluded from the data analysis; one ewe due to the presence of a persistent CL indicated by elevation of progesterone above 1ng/ml for 7 samples, three ewes did not begin cycling during the sampling period and 2 ewes were cycling prior to the onset of the sampling period.

Within the blood sampled ewes (Experiment 2), the onset of dioestrus of the first cycle of the mating season and of subsequent cycles was defined as the sample date when the progesterone level was raised above 1.5ng/ml and sustained at or above this level for at least 2 consecutive samples. The number of days from the date of the ram exposure to the onset of dioestrus was compared within treatments using a sign test to give an indication of any progression in the timing of dioestrous onset dates over the pre-mating period. Absolute cycle length was calculated as the number of days between two consecutive dioestrous onset dates as defined above and Levene's test (Minitab 13.1) was used to assess the homogeneity of the variances around the median cycle length.

The synchrony of mating of the two treatment groups was determined using a method typically adopted in analysis of menstrual synchrony known as the "last month's method" outlined in detail by Weller and Weller, (1993; 1997). Based on a technique specifically designed for the measurement of group synchrony (Wilson, 1992) the data utilised for determining synchrony was based on the onset dates of the last 2 cycles; i.e. the last cyclic onset date prior to mating and the date of mating. A mean synchrony score was calculated for each ewe as her minimal absolute difference between her onset dates of dioestrus for the pre-mating and mating cycle and those of every other ewe within that group. For example if the pre-mating and mating dioestrous onset dates for ewe 1 were 21st October and 6th November and for ewe 2 were 16th October and 4th November, then the absolute minimal absolute difference is 2. The group synchrony score was the mean of every ewe's mean synchrony score and group synchrony scores were compared using the Mann-Whitney *U* test (SPSS 11.0) in accordance with the method adopted by Weller and Weller, (1993).

This method of analysis of synchrony will indicate if the exposure of ewes to rams during the pre-mating period has resulted in greater synchrony at mating, however it gives no indication of how this synchrony may have been reached. For this purpose the homogeneity of variance between the onset of dioestrus of the first cycle of the breeding season and the date of mating were assessed using Levene's test (Minitab 13.1). The variance around the median dioestrous onset dates of the last two cycles prior to mating was also compared between treatment groups using Levene's test. The penultimate cycle prior to mating was termed cycle B and the cycle immediately prior to mating was termed cycle A. Progesterone concentration could not be used to determine the end of cycle A as plasma samples were not collected after entire rams were introduced. Therefore cycle length for cycle B was adjusted for the average number of days from the observed nadir point on the progesterone profiles to the recorded date of dioestrous onset (4.01 and 4.10 for VR and VC ewes respectively). Absolute cycle length was assessed using the Mann Whitney *U* test (Minitab 13.1).

3.3.5.2 ALL EWES

For all ewes, the median times of the onset of cycling, mating and lambing and number of lambs born per ewe (within ewes lambing to the first service) were compared using the Mann Whitney *U* test due to the abnormal distribution of the data (Minitab 13.1). Levene's test (Minitab 13.1) was used to assess the homogeneity of

variance around the median time of mating and lambing as a significant difference in the variation around the median between the two data sets indicates improved synchrony within the group with less variation around the median. The total numbers of ewes bred and lambing (X days after entire ram introduction) and the number of ewes having single, twin or multiple births were analysed using Chi Square analysis.

3.4 RESULTS

3.4.1 EXPERIMENT 1

3.4.1.1 MATING DATA

The median date of mating occurred significantly earlier within the FR ewes compared to FC ewes ($P<0001$; Table 3.1). At each daily observation the cumulative number of FR ewes mated was consistently greater than FC ewes until 15 days after entire ram introduction (at least $P<0.05$; Figure 3.1). There was less variance around the median time from entire ram introduction to mating in FR ewes (Levene's test; $P<0.001$) indicating a greater degree of synchrony in the timing of mating (Figure 3.1). There was no significant difference between groups in conception rates to first service or the numbers of ewes not mated within the first 17 days of entire ram introduction. However 64% of FR ewes not conceiving to the first service were mated during the first 24 hours of entire ram introduction compared to 9% of FC ewes ($P<0.01$).

3.4.1.2 LAMBING DATA

Within those ewes lambing to first service, FR ewes had a significantly earlier median date of lambing than FC ewes ($P<0.001$; Table 3.1). Within 8 days of the onset of lambing of each group, the divergence between groups reached significance ($P<0.05$) when 49% of FR ewes had lambed compared to 34% of FC ewes (Figure 3.1). This divergence between groups was maintained until Day 17 (At least $P<0.05$). Within FR ewes there was less variance around the median lambing date (Levene's test; $P<0.01$) than FC ewes indicating a significant compaction of the lambing period (Figure. 3.1). FR ewes had a numerically higher mean litter size than VC ewes (2.30 versus 2.20; VR and VC ewes respectively) however that there was no significant difference in the numbers of ewes having single, twin or triplet lambs. There was no significant difference between treatment groups or in the numbers of ewes lambing to subsequent services or that were barren, culled or died during the experiment (Table 3.1).

Table 3.1 Effect of repeated fence-line ram exposure on time and distribution of mating (days after ram introduction), lambing (days after the onset of lambing within each group) and fertility. (Experiment 1 $ P<0.1, *P<0.05, **P<0.01, ***P<0.001)

	FC	FR	
Number of ewes	98	94	P value
Median time from entire ram introduction to mating Days (Interquartile range)	7.00 (3.0-11.0)	4.00 (1.0-6.0)	***
Total number of ewes bred by: (%)			
Day 1	9 (9)	26 (28)	***
Day 7	49 (50)	74 (79)	***
Day 14	87 (89)	91 (97)	*
Day 17	96 (98)	93 (99)	
Ewes first marked more than 17 days after entire ram introduction (%)	2 (2)	1 (1)	
Ewes lambing to first service:	86 (88)	82 (87)	
Median number of days from entire ram introduction to lambing Days (Interquartile range)	156 (151-160)	152 (149-154)	***
Total number of ewes lambed by Day X after the onset of lambing of that group: (%)			
1	1 (1)	1 (1)	
7	22 (26)	31 (38)	$
14	60 (70)	73 (89)	**
17	68 (79)	76 (93)	*
22	86 (100)	82 (100)	
Ewes lambing to first service having:			
1 lamb	9 (10)	8 (10)	
2 lambs	51 (59)	42 (51)	
> 2 lambs	26 (30)	32 (39)	
Mean litter size (± sem)	2.20 ± 0.07	2.30 ± 0.07	
Ewes lambing to subsequent services	7 (8)	11 (13)	
Ewes that were barren, aborted or died	5 (6)	1 (1)	

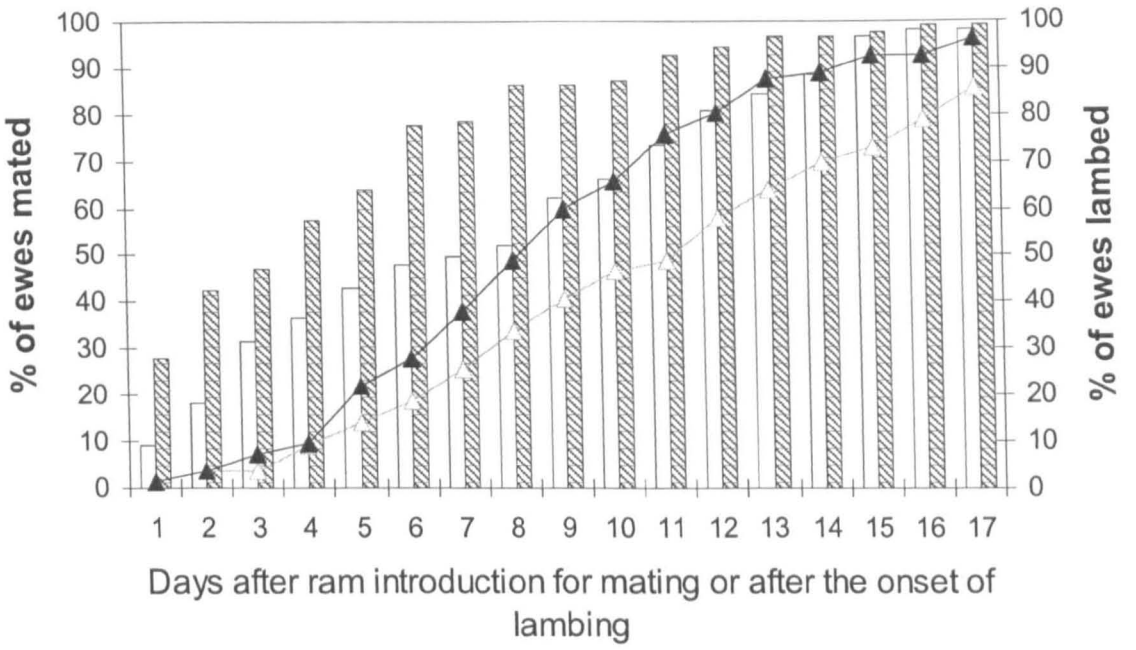


Figure 3.1 Cumulative distributions of mating (days after entire ram introduction) and lambing (days after the onset of lambing for each group) for fenceline ram-exposed ewes (mated: hatched bars n=94; lambing to first service: closed triangles n=82) and control ewes (mated: open bars n=98; lambing to first service: open triangles n=86). During the mating period raddle marks were checked daily and ewes lambing to first service were recorded as those that were not subsequently re-marked by the entire rams (confirmed by assessment of days from ram introduction to lambing).

3.4.2 EXPERIMENT 2

3.4.2.1 PRE-MATING DATA (TABLE 3.2; BLOOD SAMPLED EWES ONLY)

VR ewes had a significantly earlier median onset of cyclic activity (Table 3.2; $P<0.01$) and had a greater number of cycles prior to mating than VC ewes ($P<0.01$). These observations are likely to have been predominantly driven by the greater number of VR ewes having three oestrous cycles prior to mating (7 versus 0, $P<0.05$; VR and VC ewes respectively; Figure 3.2).

Using the progesterone data from the last two cycles prior to mating, a mean synchrony score was calculated for each group. The VR ewes had a significantly lower synchrony score than the VC ewes (2.34 versus 3.08; $P<0.01$) indicating a greater degree of synchrony at mating which is supported by the compaction of mating within the VR ewes shown in Figure 3.3. However it is also evident from Figure 3.3 that the synchrony at mating was not derived solely from a synchronous cyclic onset of the breeding season. This is shown by the progression in the cumulative distribution of dioestrous onset from the cycle after the second vasectomised ram exposure (when 100% of ewes were cycling) through to mating (Figure 3.3). This is in contrast to the VC ewes where the distribution of mating appears to have been predominantly determined by the distribution of dioestrous onset of the first cycle of the breeding season; with the greatest variation occurring after the introduction of entire rams for mating (Figure 3.4). This differential in the origin of the distributions observed at mating is supported by the persistently declining number of days from the date of the ram exposure to the onset of dioestrus over time within VR ewes, which is not evident within the VC ewes (Figure 3.5).

During the penultimate cycle prior to mating (Cycle B), VR ewes had a significantly shorter absolute cycle length than VC ewes (Table 3.2). This shorter absolute cycle length was coupled with significantly greater variance around the median cyclic length within the VR ewes indicating significantly more variability in the distribution of cycle length within VR ewes (Levene's test; $P<0.05$). This is in contrast to the cycle immediately prior to mating (Cycle A; raddle mark data adjusted by 4.01 and 4.10 for VR and VC ewes respectively) where there was no significant difference in absolute cycle length or in the variance around the median cycle length.

Within the VR ewes, the number of ewes in oestrus and thus marked by the vasectomised rams during each exposure period persistently increased over time from 0 during ram exposure 1 to 4% after ram exposure 2 and 17% after ram exposure 3. However as a proportion of ewes cycling at the time of each ram exposure the percentage of ewes in oestrus was sustained at 19% of those ewes cycling during ram exposure periods 2 and 3.

Table 3.2. Effect of vasectomised ram exposure on the onset of the breeding season and cycle characteristics of the oestrous cycles prior to entire ram introduction for mating. (Experiment 2; $ P<0.10, * P<0.05, ** P<0.01)

	VC	VR	P-Value
Number of ewes (Anovular blood sampled ewes only)	29	34	
Median onset date of the first cycle (Interquartile range)	10th October (7th – 10th October)	7th October (3rd - 7th October)	**
Number of ewes cycling on date of ram exposure			
1 (10th September)	0 (0)	0 (0)	
2 (27th September)	0 (0)	7 (21)	*
3 (14th October)	29 (100)	34 (100)	
Median cycle length (B) Penultimate cycle prior to mating (VC, n=28; VR, n=34) (Interquartile range)	17.0 (17-18)	15.5 (14-17)	**
Median cycle length (A) Cycle prior to mating (VC, n=29; VR, n=34) (Interquartile range)	15.10 (14.1-16.6)	16.01 (15.01-17.01)	

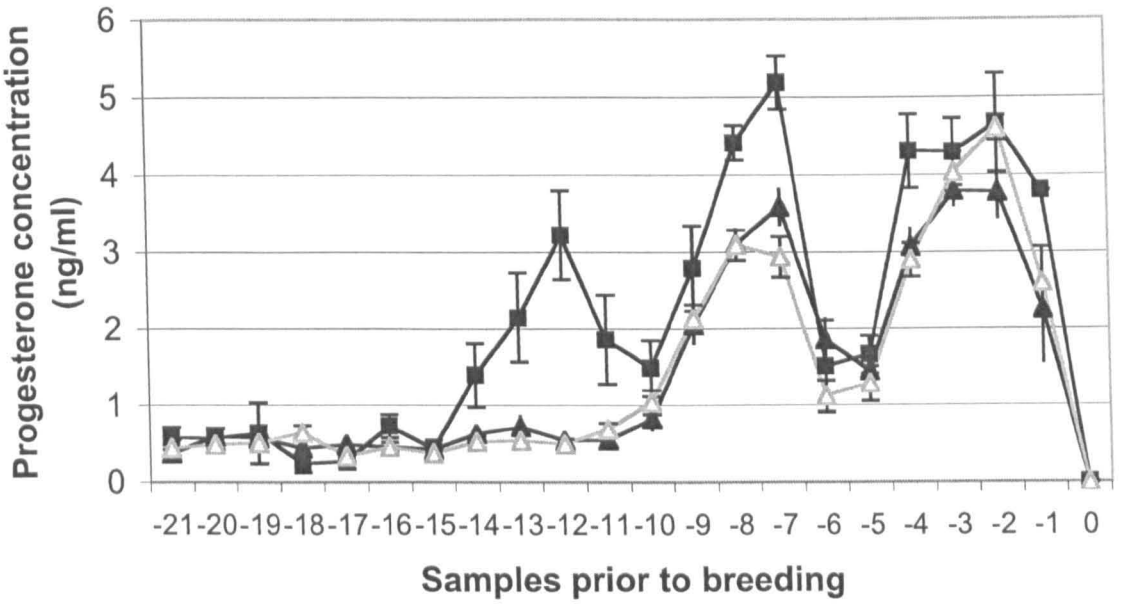


Figure 3.2. Mean progesterone profiles (± SEM) for vasectomised ram-exposed and control ewes having 2 (VR: closed triangle, n=27; VC: open triangle, n=28) or 3 (VR: closed square, n=7) oestrous cycles prior to mating. Progesterone profiles were produced for control and vasectomised ram-exposed ewes from analysis of blood samples for progesterone taken twice weekly from 2 weeks before the first ram exposure through to ram introduction for mating. Note that one control ewe had only one oestrous cycle prior to mating and progesterone data for this ewe is not shown.

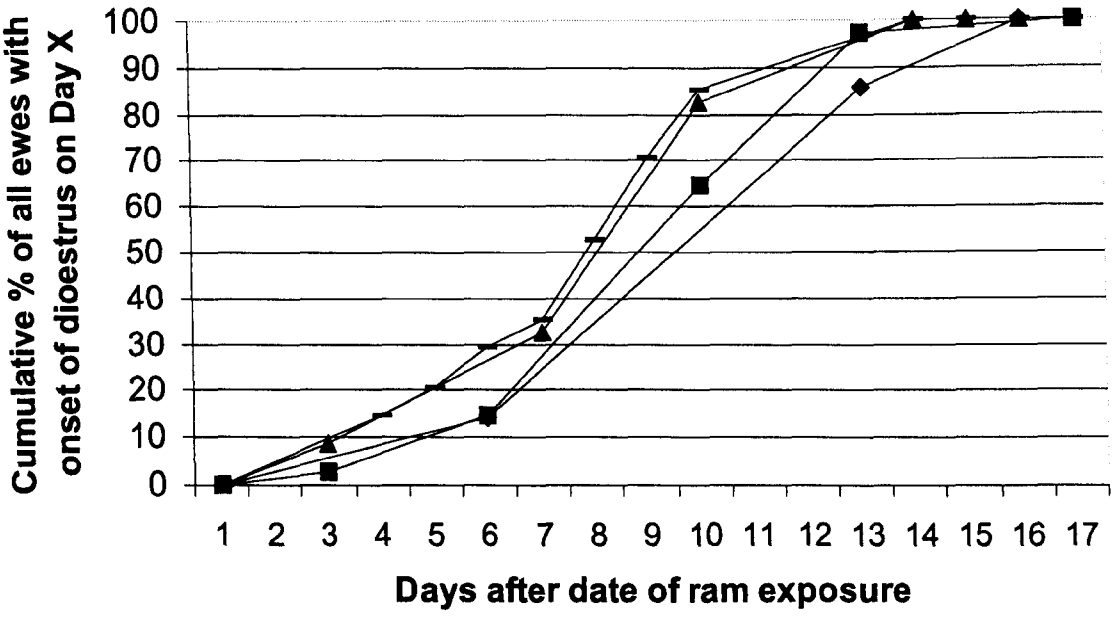


Figure 3.3. Cumulative distribution of dioestrous onset dates for VR ewes over the three cycle lengths prior to mating in terms of days after the date of each vasectomised ram exposure (Cycle C: closed diamond, n=7; Cycle B: closed square, n=34; Cycle A: closed triangle, n=34) and at mating (dash, n=34). Ewes were blood sampled twice weekly for progesterone and the onset of dioestrus was determined as the sample when progesterone concentrations rose above 1.5ng/ml. The onset date of dioestrus after mating was calculated from adjusted raddle mark data as date of marking + 4.01 days (4.01 days was derived from the mean number of days from the nadir point on the progesterone profiles to the recorded onset date of dioestrus as defined above).

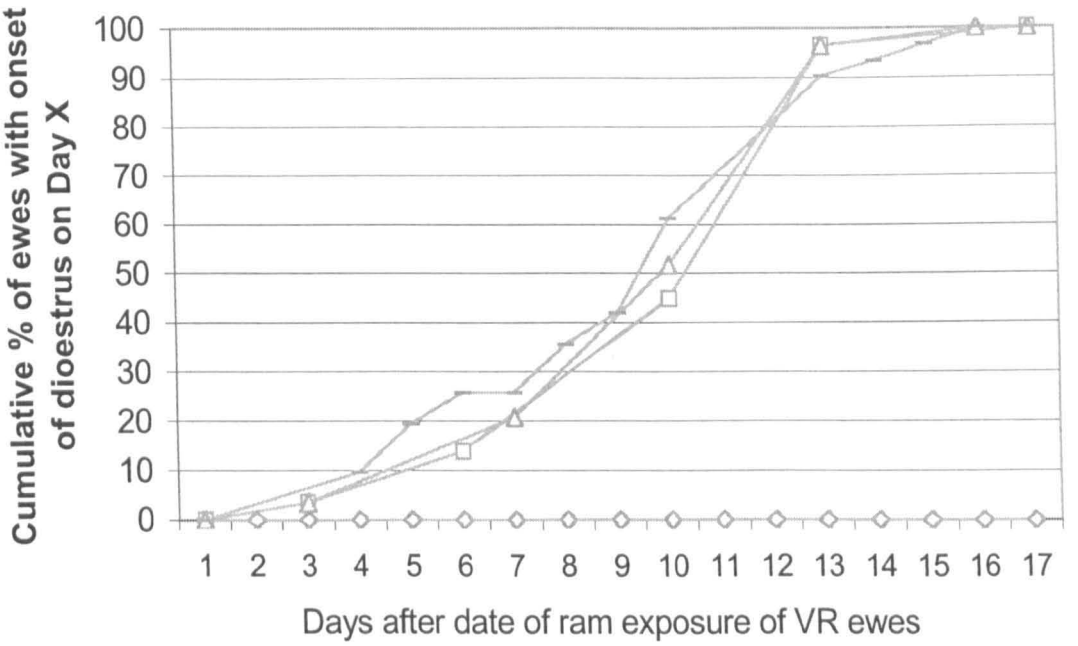


Figure 3.4. Cumulative distribution of dioestrous onset dates for VC ewes over the three cycle lengths prior to mating in terms of days after the date of the ram exposures of the VR ewes (Cycle C: open diamond, n=0; Cycle B: open square, n=28; Cycle A: open triangle, n=29) and during mating (dash, n=29). Ewes were blood sampled twice weekly for progesterone and the onset dioestrus was determined as the sample when progesterone concentrations rose above 1.5ng/ml. The onset date of dioestrus after mating was calculated from adjusted raddle mark data as date of marking + 4.10 days (4.10 days was derived from the mean number of days from the nadir point on the progesterone profiles to the recorded onset date of dioestrus as defined above).

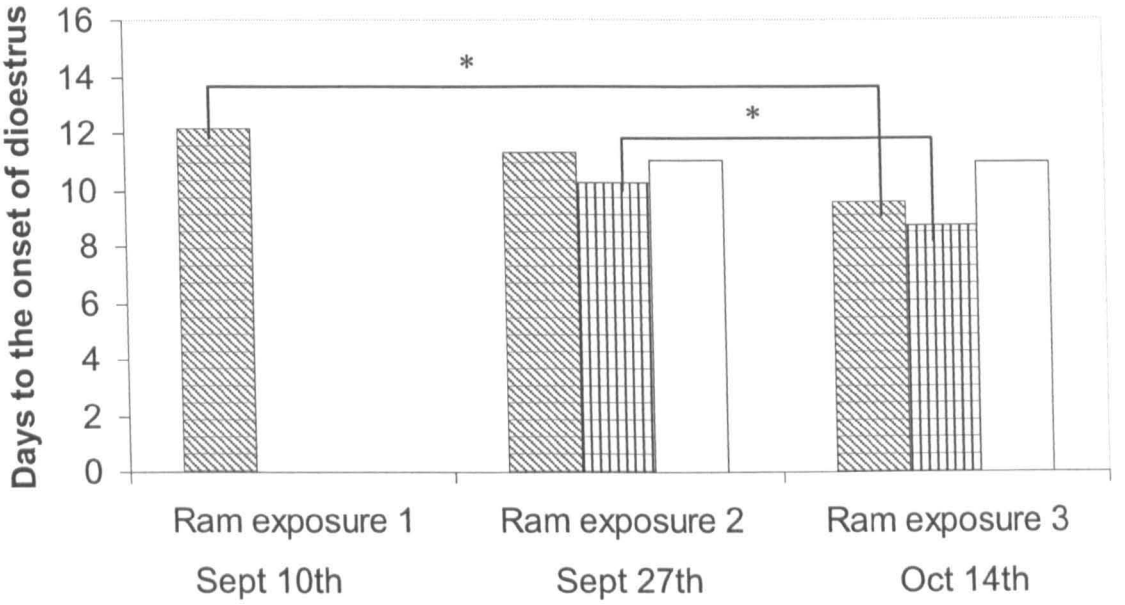


Figure 3.5. Mean number of days from the date of the ram exposures of the VR ewes to the onset of the first or subsequent oestrous cycle over the three cycle lengths prior to mating within vasectomised ram-exposed (3 cycles: hatched bars, n=7; 2 cycles: vertically hatched bars, n=28) and control ewes (2 cycles: open bars, n=28). Due to the abnormal distribution of the data, the number of days between dioestrous onset dates after each ram exposure period was compared using the Wilcoxon signed rank test. Within VR ewes having three cycles prior to mating there was a significant depression in the days from ram exposure to dioestrous onset between after ram exposure periods 1 and 3 ($P<0.05$) but not between periods 2 and 3 ($P>0.1$). Within VR ewes having two cycles prior to mating there was significant depression in days from ram exposure to dioestrous onset between ram exposure periods 2 and 3 ($P<0.05$). Within the VC ewes there was no significant depression in days from the date of the ram exposure of VR ewes to dioestrous onset ($P>0.1$).

3.4.2.2 MATING AND LAMBING DATA (ALL EWES; TABLE 3.3)

The median date of mating occurred significantly earlier within the VR ewes compared to VC ewes (Table 3.3; $P<0.001$). The cumulative number of VR ewes mated was consistently numerically greater than VC ewes from Day 1 after ram introduction (Figure 3.6) and reached and maintained significance from Days 4 to 10 after ram introduction (Table 3.3; at least $P<0.05$). VR ewes had significantly less variance around the median time of mating (Levene's test; $P<0.01$) which supports the lower synchrony score calculated from the progesterone data thus indicating a greater degree of synchrony of mating within the VR ewes.

Within those ewes lambing to first service, VR ewes had an earlier median lambing date than VC ewes (Table 3.3; $P<0.001$). The cumulative number of VR ewes lambed was consistently numerically greater than VC ewes from Day 2 after the onset of lambing and significance was reached and maintained from Days 4 to 16 of the lambing period (Figure 3.6; At least $P<0.05$). The variance around the median time from ram introduction to lambing was less for VR than VC ewes (Levene's test; $P<0.01$) indicating that a degree of the synchrony observed at mating had continued through to lambing in the VR ewes. VR ewes had a lower mean litter size than VC ewes (Table 3.3). A chi square test shows that this was due to a tendency for VR ewes to have more single lambs than VC ewes (Table 3.3; $P<0.1$). Figure 3.7 shows that with the exception of Day 2 after mating the VR ewes had a persistently lower litter size than VC ewes. However there appeared to be no significant association between the time of mating and number of lambs born. Similarly the number of lambs born was not influenced by the number of cycles prior to mating or maximum progesterone concentration when considered overall or in cycles A or B. Furthermore there was no significant difference between treatment groups in the numbers of ewes lambing to subsequent services or that were barren, culled or died during the experiment (Table 3.3).

Table 3.3. Effect of repeated exposure to vasectomised rams on the time and distribution of mating (days after ram introduction), lambing (days after the onset of lambing within each group) and fertility. (Experiment 2; $ P<0.10, * P<0.05, ** P<0.01 *** P<0.001)

	VC	VR	
Number of ewes	106	103	P Value
Median time from entire ram introduction to mating Days (Interquartile range)	6.00 (3-8)	5.00 (2-6)	***
Total numbers of ewes bred by: (%)			
Day 1	12 (11)	21 (20)	$
Day 7	63 (59)	90 (87)	***
Day 14	105 (99)	103 (100)	
Ewes first marked more than 17 days after entire ram introduction (%)	1 (1)	0 (0)	
Ewes lambing to first service:	96 (91)	93 (90)	
Median number of days from ram introduction to lambing Days (Interquartile range)	153 (151-155)	151 (149-153)	***
Total number of ewes lambed by Day X after the onset of lambing of that group: (%)			
1	2 (2)	1 (1)	
7	24 (25)	53 (57)	***
14	84 (88)	91 (98)	**
17	93 (97)	93 (100)	$
Ewes lambing to first service having: (%)			
1 lamb	10 (10)	18 (19)	$
2 lambs	65 (68)	60 (65)	
> 2 lambs	20 (21)	15 (16)	
Mean litter size	2.16 ± 0.07	1.99 ± 0.07	$
Ewes lambing to subsequent services	6 (6)	6 (6)	
Ewes that were barren, aborted or died	4 (4)	4 (4)	

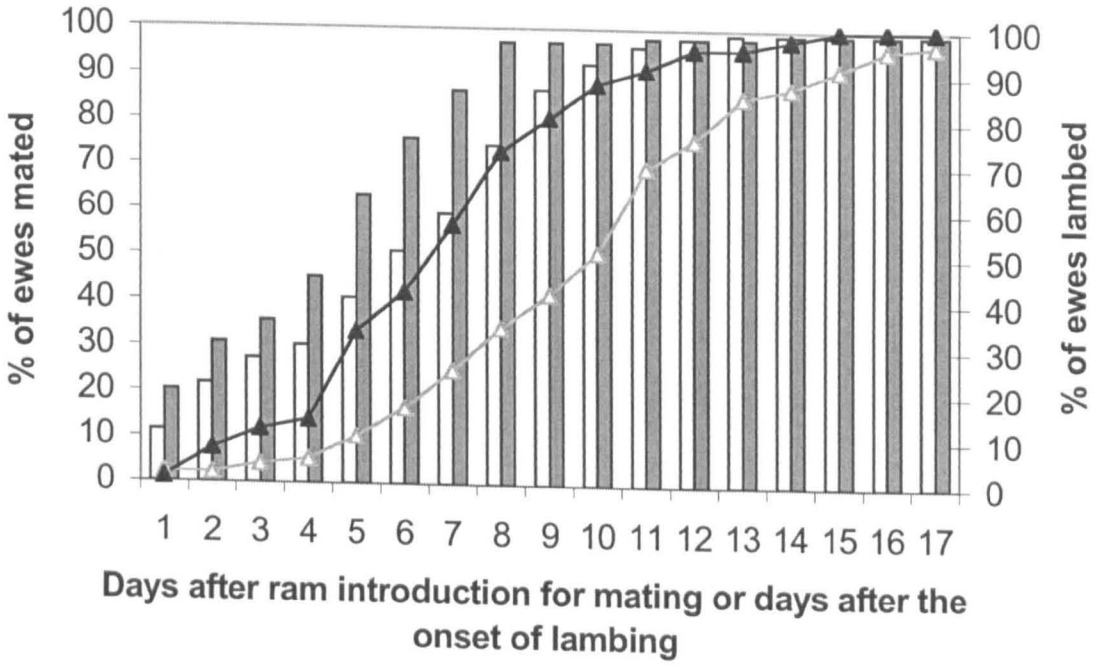


Figure 3.6 Cumulative distributions of mating (days after entire ram introduction) and lambing (days after the onset of lambing of each group) for vasectomised ram-exposed (mated: hatched bars, n=103; lambing to first service: closed triangles, n=93) and control ewes (mated: open bars, n=106; lambing to first service: open triangles, n=96). During the mating period, raddle marks were checked daily and ewes lambing to first service were recorded as those that were not subsequently re-marked by the entire rams (confirmed by assessment of days from ram introduction to lambing).

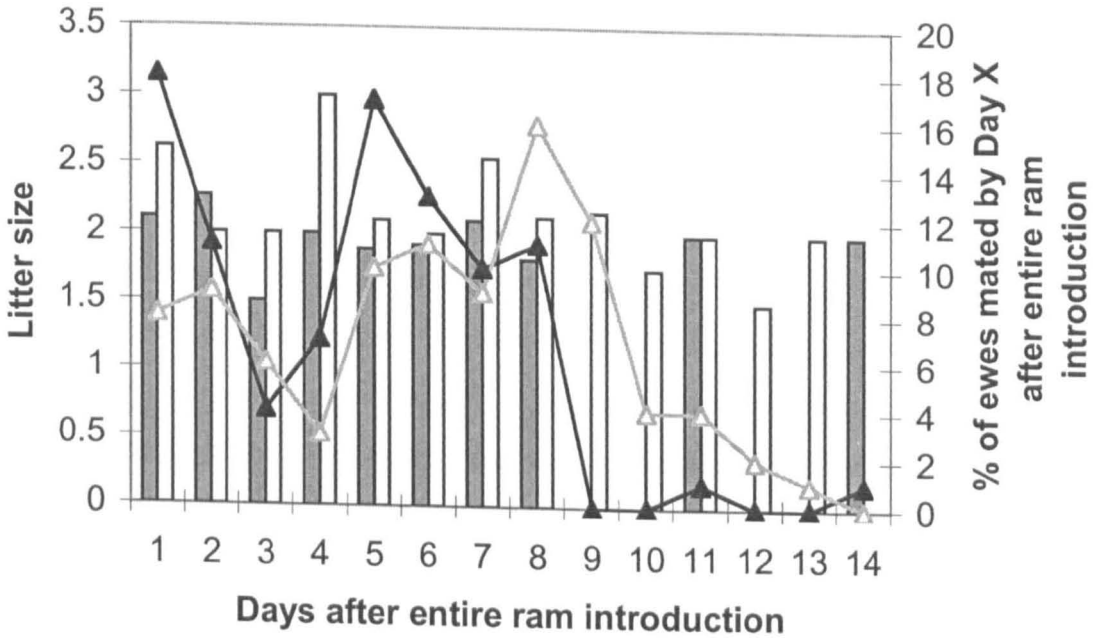


Figure 3.7. Litter size of vasectomised ram exposed (VR, hatched bars; n=93) and control (VC, open bars; n=96) lambing to first service relative to time of mating (VC; open triangles, n=96; VR; closed triangles, n=93).

3.5 DISCUSSION

Ewes exposed to both fence-line and vasectomised ram contact showed a significantly advanced and compacted distribution of both time of mating and lambing with no negative impact on conception rates to the first service. The marked effect of these repeated short duration ram exposures on the distribution of the mating period indicates that continued ram presence is not necessary to sustain an ovulatory response, a result that contradicts many assumptions within the literature. Oldham and Pearce (1983) demonstrated a depletion of the LH response within anoestrous ewes exposed to rams if the rams were removed 6 hours after introduction. Furthermore Signoret *et al.,* (1982) demonstrated a direct relationship between the duration of ram presence and the numbers of anoestrous ewes ovulating. Specifically when rams were removed after 24 hours, only 19% of ewes ovulated in response to ram introduction compared to 60% after 15 days (Signoret *et al.,* 1982). Knight (1980) demonstrated that 24 hours was a sufficient exposure period to induce an ovulatory response in anoestrous Romney ewes. However when a 24-hour exposure was applied early in the breeding season, the distributions of mating and lambing spanned weeks (Knight, 1980) rather than the compacted period of days achieved in this study. The principal assumption of a need for sustained ram presence is widely supported in the literature (Review; Martin *et al.,* 1986) and remains a focus of methodology of the majority of recent 'ram effect' studies conducted during anoestrus irrespective of breed or climate (Mexico, Merino Rambouillet: Urittia *et al.,* 2000; UK, Mule ewes: Al-Mauly *et al.,* 1991). Therefore I hypothesise that the synchrony observed in this study in ewes exposed to both the fence-line and vasectomised ram-exposed ewes is likely to be a function of the sequential repetition of the ram stimulus, isolation between exposures and most critically the proximity of the ram exposures to the natural breeding season.

Response to conventional use of the ram effect is widely accepted to be significantly affected by depth of seasonality (Martin *et al.,* 1986) and is positively correlated with the proportion of anoestrous ewes spontaneously ovulating that increases with the advancing onset of the natural breeding season (Lindsay and Signoret, 1980). The first ram exposure within both experiments in this study occurred during early September when the mule ewe is likely to be in the transitional period between anoestrus and the breeding season (Mitchell *et al.,* 1997). This transitional period is characterised by fluctuating sensitivity of the hypothalamic-hypophyseal axis to oestradiol and is accompanied by shifts in LH pulse frequency (Karsch *et al.,* 1984). Karsch (1980)

suggested that during this transitional period, sustained elevation of LH might be sufficient to induce a LH surge and the first ovulation of the breeding season. The mule ewe is capable of responding to the ram effect at this time of year with an increase in LH concentrations (Stansfield *et al.,* 1987; Al-Mauly *et al.,* 1991). Therefore I hypothesise that the timing of the periodic fence-line or vasectomised ram exposures during this transitional period may have been sufficient to induce an increase in LH concentrations that in some ewes was sufficient to induce or influence the timing of the first ovulation of the breeding season. This theory is supported by the 20% of VR ewes, which had their first oestrous cycle of the breeding season during the 17 days after the first vasectomised ram exposure of the season compared to the control ewes where no ewes ovulated during this period.

The progesterone data indicates that at the time of the second vasectomised ram exposure, 80% of the vasectomised ram-exposed ewes were still anovular. The raddle mark data indicates that within the group, 4% of the VR ewes were in oestrus during the second ram exposure period. These sexually receptive ewes may have had a direct effect on the remaining anovular ewes or an indirect effect by modulating the behaviour of the vasectomised rams during the subsequent exposure periods. The potency of oestrous females in inducing an onset of cyclic activity in anoestrous females is well documented in goats (Restall *et al.,* 1993; Walkden-Brown et al., 1993). Evidence of a similar female-to-female effect in sheep is equivocal due partly to the use of the ram for detection of oestrus (Zarco *et al.,* 1995) thus any stimulation of oestrus may merely be a reflection of a ewe-ram-ewe effect. This type of 'social facilitation' is a widely reported phenomenon in both sheep (Knight 1985, Knight *et al.,* 1998; Nugent and Notter, 1990; Gonzalez *et al.,* 1991a) and goats (Walkden-Brown *et al.,* 1993). There is considerable evidence that exposure of rams to oestrous ewes evokes an increase in LH (Gonzalez *et al.,* 1998) and testosterone (Knight *et al.,* 1998) concentrations and that alterations in these endocrine parameters affect the expression of sexual behaviour (Gonzalez *et al.,* 1991a; Rosa *et al.,* 2000). Perkins and Fitzgerald, (1994) identified a major functional role of libido and courting behaviour in the success of ram induced ovulation in anoestrous ewes thus inferring a direct link between sexual behaviour and ewe response to the ram effect. Indeed Knight (1985) identified a significant benefit in using pre-stimulated rams to induce ovulation in anoestrous ewes compared to previously un-stimulated rams. Therefore the presence of oestrous ewes during the second exposure period may have further

enhanced the potency of the ram stimulus and been associated with a stronger endocrine response to the rams. I hypothesise that this social facilitation, in combination with the timing of the second vasectomised ram exposure closer to the natural breeding season were responsible for the remaining ewes having their first oestrous cycle within 11 days of the vasectomised ram exposure period. Furthermore the comparatively fewer days from vasectomised ram exposure to the onset of dioestrus within the vasectomised ram-exposed ewes having two rather than three cycles further supports an enhancement of ewe sensitivity and/or the ram stimulus during the second vasectomised ram exposure period.

Within the vasectomised ram-exposed ewes the synchrony observed at mating does not appear to be solely due to a synchronous onset of cycling stimulated by the first, second or third ram exposure period that was then sustained through to mating as we had originally hypothesised. Within the vasectomised ram-exposed ewes there appears to be a progression in synchrony over the three successive cycle lengths prior to mating. This is indicated by the tightening of the distribution of dioestrous onset shown in Figure 3.3. This is in marked contrast to the control ewes where the synchrony attained at the onset of the breeding season is the predominant factor driving the distribution of mating as shown in Figure 3.4. Therefore this raises the question as to the origin and mechanism behind the progression in synchrony observed within the vasectomised ram-exposed ewes and whether this mechanism is also likely to be responsible for the synchrony observed in the ewes exposed only to fence-line ram contact.

Over the three sequential vasectomised ram exposures there is a progressive decline in the number of days from the date of the ram exposure to the dioestrous onset of the first or subsequent oestrous cycles as shown in Figure 3.5. As the ram exposures were timed at 17-day intervals this suggests a progression towards synchrony that once the ewes are cycling can only be mediated through a variation in cycle length. Though this is subject to some variation due to the twice-weekly blood-sampling regimen, the lack of a similar significant decline within the control ewes indicates that this is an important observation. Within a number of species the presence of the male is associated with an alteration in the pattern of oestrous cycles though there are conflicting reports of an extension and/or compaction of cycle length between species (Review; McClintok, 1983). Skinner *et al.*, (2002) found that the introduction of the

male to cyclic Springbok ewes compacted the distribution of oestrus and was associated with an extension of the cycle length i.e. a luteotrophic effect of the male. However all Springbok ewes were in their luteal phase when the males were introduced and studies in other species have shown that stage of cycle can be a critical factor to whether male has a luteotrophic or luteolytic effect (woolly opossums; Perret and M'Barek, 1991).

In several review articles a role of the ram effect during the breeding season has been somewhat dismissed due to the confounding factor of sporadic sustained elevations in progesterone within randomly cycling sheep (Pearce and Oldham, 1984; Walkden-Brown *et al.*, 1999). However Ngere and Dzakuma, (1975) identified a direct effect of ram exposure on the distribution of mating within randomly cycling aseasonal tropical ewes. Furthermore Chemineau (1983) identified a degree of synchronisation of oestrus within cyclic Creole goats that was speculated to have been driven by a luteolytic effect of the buck. However neither study show a conclusive mechanism driving this attainment of synchrony. Several studies have shown that ewes under the influence of artificial progesterone respond to ram exposure with a similar but suppressed increase in LH concentrations to that seen in untreated anoestrous ewes (Poindron *et al.*, 1980; Pearce and Oldham, 1983; Evans *et al.*, 2004). Furthermore there is evidence that this LH response advances the LH surge and ovulation post sponge removal (Evans *et al.*, 2004) with subsidiary effects on fertility parameters (Evans *et al.*, 2004; Hawken *et al.*, 2005). These observations would therefore suggest that randomly cycling ewes in their luteal phase might respond to ram exposure with an increase in LH concentrations that could have affected the follicle dynamics within that and the subsequent cycle. I propose that the isolation of ewes between exposure periods is likely to result in elicitation of an LH response during each ram exposure period because it is greater than the proposed minimal 2-week isolation period outlined by Martin *et al.*, (1986). The subsidiary effects of ram exposure whilst under the influence of progesterone may act in a similar way to that proposed by Hawken *et al.*, (2005) resulting in an earlier LH surge driven by the presence of a large follicle stimulated by the ram induced elevation in LH. Therefore this would serve to reduce the inter-dioestrous interval, effectively resulting in a compaction in cycle length.

However I propose that the effects of a ram induced elevation in LH in cyclic ewes may not only be dependent on whether the ewes are in their follicular phase or luteal

phase, but may also be dependent on the stage of their luteal phase when exposed to the ram stimulus. Nephew *et al.,* (1991) showed that progesterone levels during days 2 to 4 after mating within ewes which characteristically show "short" cycle length were higher than ewes classified as having "long" oestrous cycles (15.9 ± 0.1 versus 18.6 0.4 days; mean ± sem). Elevated levels of LH during the early luteal phase are essential for the development of a competent corpus luteum and progesterone production from the small (LH dependent) luteal cells during this early luteal phase (Niswender *et al.,* 1994). Nephew *et al.,* (1991) proposed a causal relationship between progesterone concentrations during the early luteal phase and cycle length. Therefore a ram induced increase in LH and consequently progesterone may be another possible mechanism behind the observed variation in cycle length driving the ram-exposed ewes towards synchrony at mating.

The timing of release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the predominant driving force of the timing of luteolysis and thus cycle length (Zarco *et al.,* 1988). However to my knowledge there are no direct studies into the effect of ram introduction on $PGF_{2\alpha}$ or factors affecting $PGF_{2\alpha}$ release. However though LH is luteotrophic in action during the early part of the luteal phase (Niswender *et al.,* 1994) there is some evidence of a luteolytic role of LH towards the end of the luteal phase. In the ewe, Weems *et al.,* (2003) identified an increase in secretion of $PGF_{2\alpha}$ when ewe endometrium was exposed, *in vitro*, to LH on Day 15 of the oestrous cycle. A multifunctional role of LH in luteolysis in the non-bred ewe mediated through up-regulation of LH receptors in the uterine endometrium during the late luteal phase could offer a further mechanism driving the observed variance in cycle length in ram-exposed ewes. However the levels of LH used *in vitro* to elicit this type of response are likely to be higher than that produced in response to the ram especially whilst under the influence of progesterone (Weems *et al.,* 2003; 10ng/ml, Chapter 3; pulse amplitude: 1-2ng/ml). Furthermore the LH stimulation used *in vitro* is likely to be constant rather than pulsatile, as is characteristics *in vivo*. Therefore this area requires further work to elucidate whether LH does have a role in luteolysis mediated through uterine LH receptors *in vivo* and only then could it be deduced whether a ram induced increase in LH could affect cycle length in this way.

An effect of the ram on the length of the follicular phase is more easily rationalised based on the observations that continued ram presence during the pro-oestrus and oestrus significantly advanced the time of ovulation relative to the onset of oestrus (Lindsay *et al.,* 1975). These observations are supported by those observed during an artificially induced follicular phase where ram presence post sponge removal was associated with an accelerated onset and reduced duration of oestrus (Maxwell, 1986; Romano *et al.,* 2000; 2001). Therefore I propose that a ram induced increase in LH in ewes in their follicular phase at the time of ram introduction, may have stimulated a similar advance of oestrus, the subsequent LH surge and ovulation, thus reducing the inter-dioestrous interval and cycle length.

A reduction in cycle length was not however evident within all vasectomised ram-exposed ewes and this may be an important observation in understanding the mechanism driving the progressive attainment of synchrony evident in this study. Yildiz *et al.,* (2002) proposed that the depression in LH pulse frequency observed in their study when anoestrous ewes were exposed to oestrous ewes may have been due to the stage of cycle of the stimulatory ewes. It was proposed that ewes at differing stages of their cycle may elicit stimulatory or inhibitory signals to co-ordinate the onset of the anoestrous ewe's oestrous cycles with their own (Yildiz *et al.,* 2002). This concept of enhancement and suppression of the ovarian cycle in rodents is comprehensively reviewed by Mclintok *et al.,* (1983) but remains a contentious issue due to natural variation in cycle length and further controversy over the method of cycle measurement (Schank, 2000; 2001). Studies into the effects of cyclic ewes on the oestrous cycles of their flock mates is lacking in the literature and is an area that requires further investigation to determine if it is an active mechanism driving the synchrony observed in this study. Another possible factor that may explain the lack of a uniform effect of the ram exposure within the vasectomised ram exposed ewes is the role of dominance. Alvarez *et al.,* (2003) found that high-ranking goats ovulated and conceived earlier in response to the buck effect and deduced this to be due to a monopolisation of time with and proximity to the buck. Within ewes, the role of dominance and social hierarchy appears to be relatively breed dependent (Squires and Daws, 1975) and is positively correlated with liveweight (Lobato and Beilharz, 1979). Therefore I speculate that it is possible that there may have been a hierarchy driven differential within the ram-exposed ewes in the level of intensity and endocrine response to the ram exposures. Thus as I am proposing that the alterations in cycle

length may have been driven by a variable endocrine response to the rams, this may have affected their distribution of cyclicity during the pre-mating period.

The ram effect is not driven solely by pheromonal cues and maximum ovulatory responses to the ram effect during anoestrus are widely accepted to be associated with exposure to the full range of exteroreceptive stimuli (Pearce and Oldham, 1988). Therefore it is predictable that the mating period of the vasectomised ram-exposed ewes (Experiment 2) was distributed over a compacted number of days compared to the fenceline ram-exposed ewes (Experiment 1). Progesterone data from the control ewes in Experiment 2 indicates that the distribution observed at mating originates from a relatively compact distribution of the onset of cyclic activity. I hypothesise that this divergence in the control ewes is due to the blood sampling protocol in Experiment 2. Ewes are very susceptible to stress and in particular the handling element of the blood sampling (van Lier *et al.*, 1998). There is much evidence that stress such as that induced by transport can stimulate ovulation in anoestrous ewes (Braden and Moule, 1964). The control ewes within Experiment 1 were not handled during the pre-mating period and thus we hypothesise that the divergence in the distributions of mating in the control ewes within Experiment 2 was due to a stress induced, more synchronised onset of cycling. This theory is supported by the progesterone data for the control ewes that indicates that the synchrony evident at the onset of the breeding season was sustained at that level through to mating.

The vasectomised ram-exposed ewes tended to have a lower litter size than control ewes; a trend that was not evident within the fenceline ram-exposed ewes. However due to the critical necessity for group to group separation, the confounding factor of nutritional differences in pastures (both between locations and years) prevents any discussion of an effect of ram exposure per se, as nutrition is a primary influential factor on litter size (Review; Gunn, 1982). This area would require further investigation to determine if it is a significant observation.

In summary, both short term fenceline ram-exposure and vasectomised ram-exposure repeated at three 17-day intervals from the transition into the heart of the breeding season, resulted in a compaction of mating and subsequent lambing. The full complement of male sensory cues provided by the use of vasectomised rams during the 24-hour exposure periods resulted in a more synchronous distribution of mating

and lambing than fenceline ram contact alone. I propose that the synchrony observed at mating is a function of the timing of the ram exposures in the transition into the breeding season, (when the hypothalamic-hypophyseal axis is waning in sensitivity to the negative effects of oestradiol) and the repetition of the exposures once the ewes are cycling. However the ram-induced synchrony does not appear to be driven solely by a synchronous onset of the breeding season. I propose that the observed synchrony develops over time through an effect of the ram-induced endocrine response on ewes at different stages of their cycles thus concentrating the eventual mating period over a reduced number of days.

4. INVESTIGATION INTO THE EFFECTS OF DURATION AND FREQUENCY OF PERMANENT AND INTERMITTENT VASECTOMISED RAM EXPOSURES DURING THE TRANSITION INTO BREEDING SEASON ON THE DISTRIBUTION OF MATING AND LAMBING.

4.1 ABSTRACT

Continuous ram presence is typically deemed necessary for maintenance of an endocrine response to ram introduction. However within Chapter 3, I have shown the potential for short term (24 hour) ram exposure periods to influence the onset of the breeding season and the distribution of mating. The main aim of this experiment was to compare the efficacy of intermittent and continuous ram exposures at synchronising the distribution of mating and lambing. During August, mule ewes were allocated to one of four groups; RVR ewes (n=113) were exposed to vasectomised rams for 24 hours on Days 0, 17 and 34 of the experiment; SVR ewes (n=109) were exposed to vasectomised rams for 24 hours on Day 0 and subsequently isolated from ram contact; PVR ewes (n=104) were introduced to vasectomised rams on Day 0 and they remained with the ewes for the duration of the pre-mating period; NVR ewes (n=113) were maintained as outlined for PVR ewes but with rams replaced every 17 days during the pre-mating period. Subsets of each group (n=22) were blood sampled twice weekly to monitor their pre-mating progesterone profiles. At mating, raddled entire rams were introduced 16 days after the last ram exposure and raddle marks were recorded daily. Ewes maintained in continuous ram presence had an earlier onset of the breeding season (At least $P<0.05$) and more ewes had three oestrous cycles prior to mating than intermittent ram exposed ewes ($P<0.05$). Synchrony scores were calculated for each group and indicated a greater degree of synchrony at mating in ewes maintained in continuous ram presence when compared to intermittent ram exposed ewes ($P<0.001$). Within the continuously ram exposed ewes, PVR ewes had a significantly earlier median date of mating than NVR ewes ($P<0.01$). Within the intermittent ram exposed ewes there was no difference in the median date of mating or in the variance around the median. However the significantly different synchrony scores between these groups ($P<0.05$) indicates a difference in the pattern of the distribution of mating in SVR and RVR ewes. Within

ewes lambing to the first service, RVR ewes had a significantly lower litter size than SVR ewes. The variation in the patterns of mating observed in this study infers the potential for tailoring the pattern of mating towards the most desirable distribution of lambing for an individual farmer.

4.2 INTRODUCTION

The proportion of ewes ovulating in response to the ram effect is positively correlated with the proximity of the onset of the natural breeding season (Lindsay and Signoret, 1980). Furthermore ewes stimulated to ovulate by the ram effect towards the end of their non-breeding season have a greater propensity to continue to cycle regularly and maintain cyclicity through to the next anoestrus (Oldham and Cognie, 1980; Rosa and Bryant, 2002). Within Chapter 3, I successfully compacted the period of mating and lambing using repeated 24-hour fenceline and vasectomised ram exposure periods, repeated every 17 days from the transition into the core of the breeding season. The first ram exposure was timed in early September and within the ewes exposed to the vasectomised rams, 20% of ewes ovulated during the 17 day period after this first ram exposure period and maintained regular oestrous cycles through to mating. However in the absence of further ram contact, what effect would these ram-stimulated ewes have on the onset of cyclic activity of the remaining anovular ewes. In the current study, I propose to compare the efficacy of a single 24-hour ram exposure period (timed at the same point relative to the onset of the breeding season as the first ram exposure period in Chapter 3) with the repeated short-term ram exposure strategy developed in Chapter 3.

Continued ram presence is typically deemed necessary for an optimum proportion of anoestrous ewes to ovulate in response to the ram effect (Oldham and Pearce, 1983). It is proposed that the continuous ram presence sustains a high concentration of LH resulting in a larger proportion of ewes having an LH surge and ovulating in response to the ram effect. Though in Chapter 3, I obtained a good level of synchrony at mating using repeated 24 hour ram exposures, the proportion of ewes that ovulated within 17 days of the first exposure (20%) is low when compared to responses to conventional application of the ram effect (Merino, 40-100%; Signoret, 1990). Therefore I propose that continued ram presence over the same pre-mating period (Early-September to November) will increase the proportion of ewes ovulating and remaining cyclic after the initial ram introduction compared to ewes exposed to a single or repeated 24 hour ram exposure strategy.

Within ewes ovulating in response to the ram effect a large proportion revert to an anovulatory state even when maintained in continued presence of the ram (Pearce and

Oldham, 1984). This led to the suggestion that the ewe becomes refractory or habituated to the ram stimulus (Martin *et al.*, 1986). This concept of habituation is supported by the initial proposal by Underwood *et al.*, (1944) that ewes must undergo a period of isolation to respond to the ram effect. Indeed ewes maintained in constant ram presence have a comparable onset of the breeding season to anoestrous to ewes isolated from the ram stimulus (Riches and Watson, 1954). However more recent work has shown that it is the novelty of the ram stimulus rather than the ram presence per se that is the predominant factor in determining the response of ewes to the ram effect (Pearce and Oldham, 1988; Cushwa *et al.*, 1992). Therefore I propose that by intermittently removing and replacing the rams with different rams (thus maintaining the novelty of the ram stimulus) I will be able to alter the pattern and synchrony of mating. Furthermore the 24-hour ram exposures used in Chapter 3 appeared to affect the dynamics of cycle length once ewes were cycling. Therefore I propose that renewing the ram stimulus at the same 17 days interval as applied in Chapter 3, I may further affect cyclic distribution over and above that of continuous ram presence alone.

This aim of this experiment was to compare the efficacy of the repeated short term vasectomised ram exposure strategy developed in Chapter 3 as a method of oestrus synchronisation with other frequencies and durations of ram exposure during the pre-mating period. The first comparison is made with a single 24-hour ram exposure to determine if any synchrony can be derived and maintained in response to a single short duration ram exposure during the transition into the breeding season. The second and third comparisons were made with ewes permanently exposed to vasectomised rams for the duration of the pre-mating period. Within one of these groups, the vasectomised rams were removed and replaced with novel rams at 17-day intervals to investigate the role of novelty and possible habituation to the ram stimulus. Specifically I aimed to compare the levels and attainment of synchrony at mating and lambing and any effects on conception rates and litter size.

4.3 MATERIALS AND METHODS

4.3.1 ANIMALS AND EXPERIMENTAL PROCEDURES

The study was conducted at Cockle Park Research Farm, Northumberland (55°13'N), using multiparous mule ewes (Swaledale x Bluefaced/Border Leicester) which had been previously isolated (not within 500m) from ram contact. During August, ewes were assigned to a repeated vasectomised ram exposure group (RVR n=113), a short duration single vasectomised ram exposure groups (SVR; n=109), permanent vasectomised ram exposure group (PVR; n=104) or novel vasectomised ram exposure group (NVR; n=113) balanced for age and parity.

Repeated ram exposed ewes (RVR)

RVR ewes were exposed to vasectomised rams (n=3) for 24 hours on Days 0 (September 11th), 17 and 34 of the experiment. The timing of the first and subsequent ram exposure periods is the same as that used in Chapter 3. Raddle marks were recorded at the end of each exposure period and raddle colour was changed before the next exposure period to monitor continued or first incidence of oestrus. Within the RVR ewes, novel vasectomised rams were used for each exposure period to permit repetition of the novelty of each exposure.

Single ram exposed ewes (SVR)

SVR ewes were only exposed to raddled vasectomised rams once (n=3) for 24 hours on Day 0 (September 11th). Raddle marks were checked at the end of the exposure period to monitor the incidence of oestrus. Ewes were then isolated from ram contact for the remainder of the pre-mating period.

Permanent ram exposed ewes (PVR)

Raddled vasectomised rams (n=3) were introduced to PVR ewes on Day 0 of the experiment (12th September, one day after the SVR and RVR ewes to permit rotation of vasectomised rams) and remained with the ewes for the duration of the pre-mating period. The raddle colour was changed at 17-day intervals on Days 17 and 34 and raddle marks were checked twice weekly to monitor the distribution of the onset of the breeding season and the subsequent timing of oestrus.

Novel ram exposed ewes (NVR)

Raddled vasectomised rams (n=3) were introduced to NVR ewes on Day 0 of the experiment (12th September) and remained with the ewes for the first 17 days of the experiment. On Day 17 they were removed and replaced with novel vasectomised rams (n=3). Novel rams were defined as vasectomised rams that hadn't been in contact with the NVR ewes for a minimum period of 2 weeks. These vasectomised rams remained with the ewes for a further 17 days until Day 34 when they were removed and replaced with another set of novel raddled rams (n=3) that remained with the NVR ewes until mating. Raddle colour was different for each set of novel vasectomised rams and raddle marks were checked twice weekly to monitor the distribution of the onset of the breeding season and subsequent timing of oestrus.

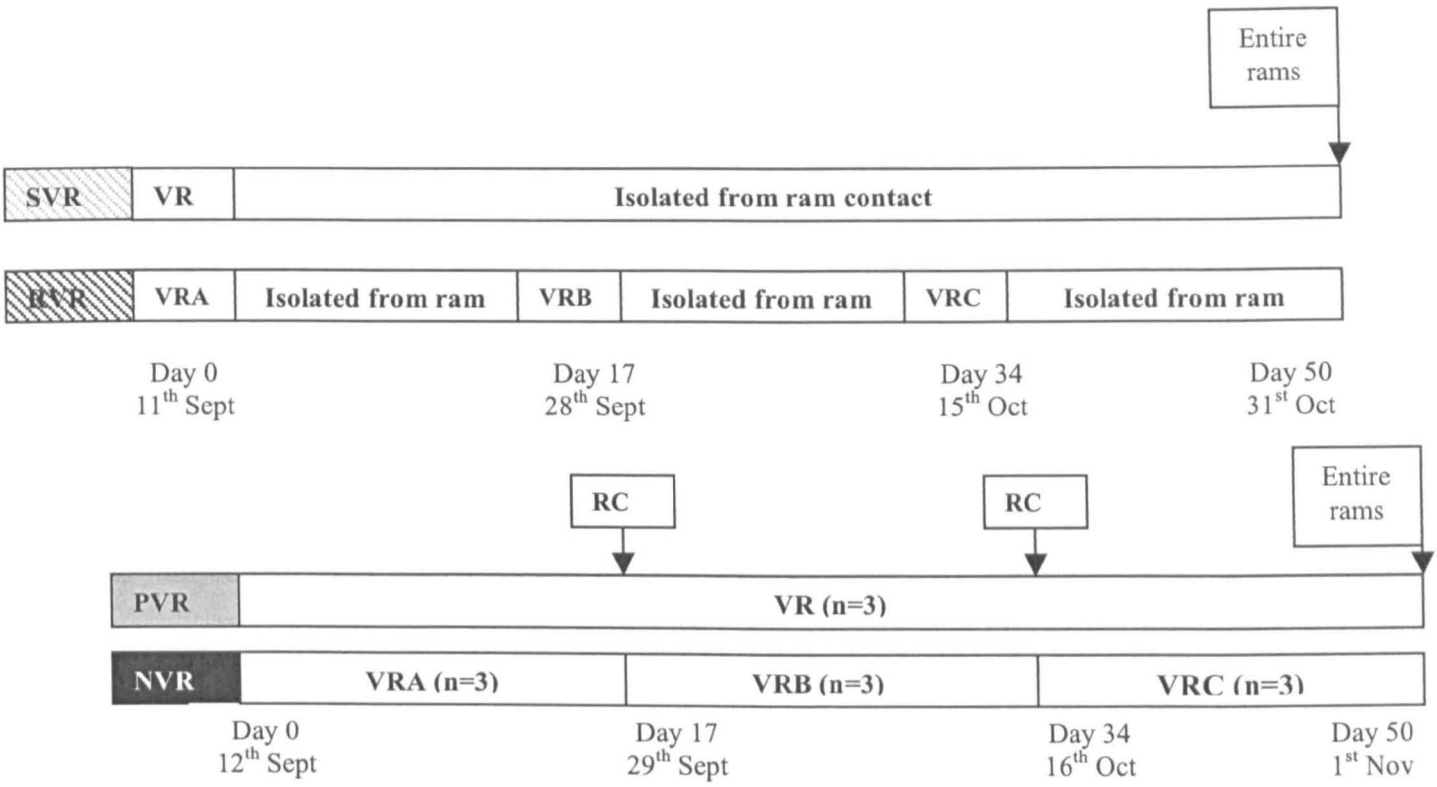


Figure 4.1 Protocol Diagram

VR Vasectomised rams (n=3) used for 24-hour exposure of SVR ewes after which they were introduced to PVR ewes and remained with these ewes for the duration of the pre-mating period. The raddle colour indicated above as RC was changed every 17 days.

VRA

VRB

VRC

The vasectomised rams (n=3) used for the three 24 hour exposures of RVR ewes were removed from RVR ewes and introduced to NVR ewes at 17 day intervals at a one day delay to permit this rotation of the rams. Each set of vasectomised rams replaced the previous set. A different raddle colour was used on each set of vasectomised rams.

RC The raddle colour on the harness of the ram was changed

Entire rams Raddled entire rams (n=4 per group) were introduced to all groups 16 days after the onset of the final ram exposure period.

4.3.1.1 MATING

The groups of ewes were not mixed at mating to avoid any effect of the number of oestrous ewes within one treatment affecting the sexual activity of the entire rams and thus possibly the distribution of mating within another treatment. Therefore to avoid any effect of the ram on the fertility parameters, entire rams were libido tested and were allocated between treatment groups according to libido score. Raddled entire rams (Suffolk, n=8 Texel, n=8) were introduced for mating (1 ram: 25 ewes) on 31st October to SVR and RVR ewes and 1st November to PVR and NVR ewes to permit introduction of entire rams on the same day relative to the last vasectomised ram exposure period (16 days). Raddle marks were recorded daily to identify the timing and numbers of ewes mated during the first oestrous cycle and then recorded weekly for the subsequent 34 days. Raddle colour was changed on Days 14 and 32 after entire ram introduction to permit identification of ewes not conceiving to the first service. Ewes were maintained in accordance with commercial farm practice until lambing when the number of lambs and date of lambing were recorded.

4.3.2 BLOOD COLLECTION

Blood samples (5ml) were collected twice weekly by jugular venepuncture (Vacutainer, Becton-Dickinson Limited, Coventry) from a subset of SVR (n=22), RVR (n=22), PVR (n=22) and NVR ewes (n=22). Sampling commenced approximately 2 weeks prior to the first vasectomised ram exposure on Day -13 (SVR and RVR) and Day -15 (PVR and NVR) and continued until to Day 50 (31st October). The sampling regime was necessary to establish whether the ewes were anovular prior to the first vasectomised ram exposure and to monitor the progesterone profiles generated in response to the different frequencies and duration of vasectomised ram exposures during the pre-mating period. Blood samples were centrifuged within 24 hours at 3000 rpm for 20 minutes. Plasma was decanted into duplicate plastic tubes (Sarstedt Ltd, Leicester, UK) that were capped, immediately frozen and stored at -20°C until analysis.

4.3.3. HORMONE ANALYSIS

Progesterone concentrations were measured in duplicate using a commercial ELISA kit (Ridgeway Science Ltd, Gloucester, UK) as outlined in Chapter 3. Mean intra-assay and inter-assay coefficients of variation for low (1.46ng/ml), medium

(2.31ng/ml) high (7.00ng/ml) plasma samples were 7.5%and 14.6%, 5.2%and 10.3% and 5.7% and 12.8% respectively. The limit of sensitivity of the assay was 0.2 ng/ml.

4.3.4 DATA ANALYSIS

4.3.4.1 BLOOD SAMPLED EWES ONLY

Blood sampled ewes were initially assessed for their anovular or cyclic status. A ewe was classified as cyclic prior to ram introduction if progesterone (during the four samples prior to the first ram exposure period) was elevated above 1.5ng/ml for at least 2 consecutive samples. Only data from ewes that were anovular at the time of the first ram exposure were used in the analysis. Within the four treatment groups, 2 SVR, 2 RVR, 3 PVR and 1 NVR ewes were cycling prior to the first ram exposure period and were thus excluded from further analysis. Due to the abnormal distribution of the data, non-parametric analyses were used throughout, with the exception of analysis of maximum progesterone concentrations that was normally distributed and thus analysed by parametric analysis.

Within the blood sampled ewes, the onset of dioestrus of the first oestrous cycle of the breeding season and of subsequent oestrous cycles was defined as the sample date when the progesterone level exceeded the mean of the previous samples by two standard deviations and was sustained at or above this level for at least 2 consecutive samples. The onset of each subsequent oestrous cycle was determined as the sample date when the progesterone level again exceeded the calculated threshold of the first oestrous cycle. Cycle length was calculated as the number of days between the onset dates of dioestrous of successive oestrous cycles.

Cyclic onset date and cycle length were firstly compared between treatments using Kruskal Wallis test (Minitab 13.1). The cycle immediately prior to mating was termed cycle A and the penultimate cycle prior to mating was termed cycle B. Where ewes had three oestrous cycles prior to mating this third cycle was termed cycle C. Progesterone concentration could not be used to determine the end of cycle A as plasma samples were not collected after entire rams were introduced. Therefore cycle length for cycle A was adjusted for the average number of days from the observed nadir point on the progesterone profiles to the recorded date of dioestrous onset (4.72, 4.52, 4.11, 5.06 for NVR, PVR, RVR and SVR ewes respectively). Where a

significant difference was detected in cyclic onset or cycle length, each treatment group was then compared by Mann Whitney *U* test (Minitab 13.1) to assess the source of this difference between groups. Levene's test (Minitab 13.1) was used to assess the homogeneity of variance around the median cycle length between treatments over the three cycles prior to mating. The number of days from the date of the ram exposure to the onset of dioestrus was compared between cycles A, B and C within treatments using a Wilcoxon Signed Rank test (Genstat 5 Release 3.2) to give an indication of any increase or decrease in the time of dioestrous onset over the pre-mating period.

Maximum progesterone concentrations over the three oestrous cycles prior to mating were analysed using General Linear Model (Minitab 13.1) to examine the effect of treatment, cycle and any interaction between the two on the maximum level of progesterone. Within PVR and NVR ewes, maximum progesterone over the three oestrous cycles prior to mating were compared using a Paired T-test. This analysis could not be conducted in the SVR and RVR ewes due to the requirement of an equal number of ewes within each cycle and the varying numbers of ewes in these treatment groups having 1, 2 or 3 oestrous cycles prior to mating.

Further analysis was undertaken to determine the level of synchrony of each treatment group at mating. The synchrony of mating of the treatment groups was determined using a method typically adopted in analysis of menstrual synchrony known as the "last month's method" (Weller and Weller, 1993; 1997) that is outlined fully in Chapter 3. The group synchrony scores were initially compared between the four treatment groups using the Kruskal Wallis test (Minitab 13.1). Where a significant difference was detected, each treatment group was then compared using the Mann-Whitney *U* test (Minitab, 13.1).

4.3.4.2 ALL EWES

After ram introduction to the PVR and NVR ewes, raddle mark data was collected twice weekly to monitor the distribution of oestrus during the pre-mating period. The number of ewes marked twice by the vasectomised rams prior to mating accounted for 87% and 81% of PVR and NVR ewes respectively. Within these ewes the number of days from the date of novel ram introduction (or date of raddle colour change) to marking by the vasectomised rams or entire rams were compared within treatment

groups using Wilcoxon Signed Rank test (Genstat 5, version 3,2) and between PVR and NVR ewes by Mann Whitney *U* test (Minitab 13.1). The number of days after ram introduction to mating was adjusted by 1 day to allow for the 16 rather than 17-day interval between entire ram introduction and the third ram exposure period. Furthermore as raddle mark data for mating were collected daily rather than the twice-weekly frequency during the pre-mating period, the mating data was further adjusted to represent ewes marked on Days 4, 7, 10, 14 and 17 to mimic the previous frequency of recording of raddle marks.

For all ewes, the times of mating and lambing were initially compared using the Kruskal Wallis test (Minitab 13.1) to detect if there was any difference between treatments. Where a significant difference was detected, treatment groups were compared by Mann Whitney *U* test (Minitab 13.1) to detect the origin of the difference. Levene's test (Minitab 13.1) was used to assess the homogeneity of variance around the median time of mating and lambing. This test was used as a significant difference in the variation around the median between data sets indicates improved synchrony within the group with less variation around the median. The total numbers of ewes mated and lambing (X days after entire ram introduction) and the number of ewes having single, twin or multiple births were compared by Chi Square analysis (Minitab 13.1).

4.4 RESULTS

4.4.1 PRE-MATING DATA (TABLE 4.1)

The progesterone data indicates that the NVR ewes and PVR ewes had a significantly earlier median onset of cyclic activity than RVR ewes (Table 4.1; $P<0.01$). NVR ewes also had a significantly earlier onset of cyclic activity than SVR ewes (Table 4.1; $P<0.05$). However although PVR ewes had an earlier onset of cyclic activity than SVR ewes this difference was not significant (Table 4.1; $P<0.1$). These differences in onset date of cyclic activity resulted in a greater number of NVR and PVR ewes having three oestrous cycles prior to mating (100% and 100% versus 40% and 25%, $P<0.05$; NVR and PVR versus SVR and RVR ewes respectively). There was no significant difference between NVR versus PVR ewes and RVR versus SVR ewes in the median onset of cyclic activity (Table 4.1; $P>0.05$).

Using the progesterone data from the last two cycles prior to mating, a mean synchrony score was calculated for each treatment at mating. NVR and PVR ewes had a significantly lower mean synchrony score than both RVR and SVR ewes (1.06 and 1.06 versus 2.64 and 3.42; $P<0.001$). RVR ewes had a significantly lower synchrony score than SVR ewes ($P<0.05$) indicating a greater compaction in the period of mating in RVR ewes. There was no difference between PVR and NVR ewes in their synchrony scores at mating.

The ranking of synchrony scores in blood sampled ewes from the lowest (indicating the greatest degree of synchrony at mating) in NVR and PVR ewes, through to the highest (thus least degree of synchrony at mating) within the SVR ewes, is in accordance with the observed distribution of mating of all ewes within the treatment groups (Figure 4.12). The absence of any difference in the synchrony score of PVR and NVR ewes, in contrast to the visible difference in the distributions of mating, is due to the different median times of mating rather than differences in the actual distribution of mating. This is clearly illustrated by the progressive deviation in the pattern of raddle mark data of PVR and NVR ewes over the three cycles prior to mating (Figure 4.2). Furthermore closer examination of the cumulative distribution of raddle mark data of NVR ewes shows a progressive shift in the location of the slope rather than a steepening of the slope over the three 17 day periods prior to mating and after entire ram introduction for mating (Figure 4.3). In contrast there is little variation in the location or slope of the cumulative distribution of raddle mark data over the three cycles prior to mating and at mating within the PVR ewes (Figure 4.4). This is further supported by the shorter time interval from vasectomised ram introduction to raddle marking in NVR ewes after the third ram exposure period (Figure 4.5; $P<0.05$) but not the after first and second ram exposure period. This observation supports the above evidence that the divergence in the median time of mating between the two groups that developed over time (Figure 4.5).

Figure 4.6 shows the mean progesterone profiles for the blood-sampled subset of PVR and NVR ewes; all of which had three oestrous cycles prior to mating. Figures 4.7-4.8 illustrate the profiles for SVR and RVR ewes having three and two oestrous cycles prior to mating. However there was no significant difference between treatments in median cycle length or the variance around median cycle length over cycles A, B or C

prior to mating (Table 4.1; P>0.1 Figures 4.9-4.11). However I propose that the twice weekly sampling regime was not sufficient to detect the subtle changes in oestrous cycle length that are likely to be responsible for the shift in the median time of mating between the NVR and PVR ewes.

Ewes maintained in continuous presence of the vasectomised rams (PVR and NVR) had higher maximum progesterone concentrations than ewes exposed intermittently to rams (SVR and RVR; P<0.001, Figures 4.9-4.11). There was an overall effect of time on the maximum progesterone concentrations during the oestrous cycle that increased significantly over the three cycles prior to mating (P<0.05, Figures 4.9-4.11). Though there was no interaction between treatment and cycle relative to the onset of the breeding season, NVR ewes had a significantly greater maximum concentration during cycle B (2^{nd} cycle prior to mating) than during the cycle C (3^{rd} cycle prior to mating; 4.10 ± 0.18 versus 4.65 ± 0.20; P<0.05). Within the PVR ewes there was no significant increase in maximum progesterone concentrations over the three cycles prior to mating (4.12 ± 0.20 versus 4.37 ± 0.23; P>0.1). A similar analysis could not be conducted on the maximum progesterone data for the SVR and RVR ewes for reasons outlined in section 4.3.4.1.

Table 4.1. Effect of different duration and frequency of exposure to vasectomised rams on the time the onset of the first dioestrous of the breeding season, time of mating and oestrous cycle characteristics **(blood sampled ewes only)**. Within rows, significant differences are indicated by different superscripts (P<0.05).

	SVR	RVR	PVR	NVR
Number of ewes	20	20	19	21
Cyclic onset				
Median days from date of first ram exposure to cyclic onset (Interquartile range) Corresponding date	15.00ac (8.0-33.0) 26th September	22.00a (15.0-29.0) 3rd October	10.00bc (10.0-13.0) 22nd September	10.00bd (10.0-11.5) 22nd September
Oestrous cycle length over the three cycles prior to mating				
Median cycle length (C) Three cycles prior to mating (Interquartile range) **Only** ewes having **three** oestrous cycles prior to mating	17.0 (14-17) (n=8)	18.0 (14.0 – 21.0) (n=5)	17.0 (17.0 – 21.0) (n=19)	17.0 (14.0-17.0) (n=21)
Median cycle length (B) Penultimate cycle prior to mating (Interquartile range) Ewes having **two** or **three** oestrous cycles prior to mating	17.0 (15.5 – 18.0) (n=17)	17.0 (14.0 -18.0) (n=18)	18.0 (14.0– 18.0) (n=19)	18.0 (14.0-18.0) (n=21)
Median cycle length (C) Cycle prior to mating (Interquartile range) Adjusted raddle mark data **All ewes**	16.14 (15.1-18.1) (n=20)	16.06 (15.1 –18.1) (n=20)	16.22 (14.7 – 17.5) (n=19)	16.52 (14.5-17.5) (n=21)

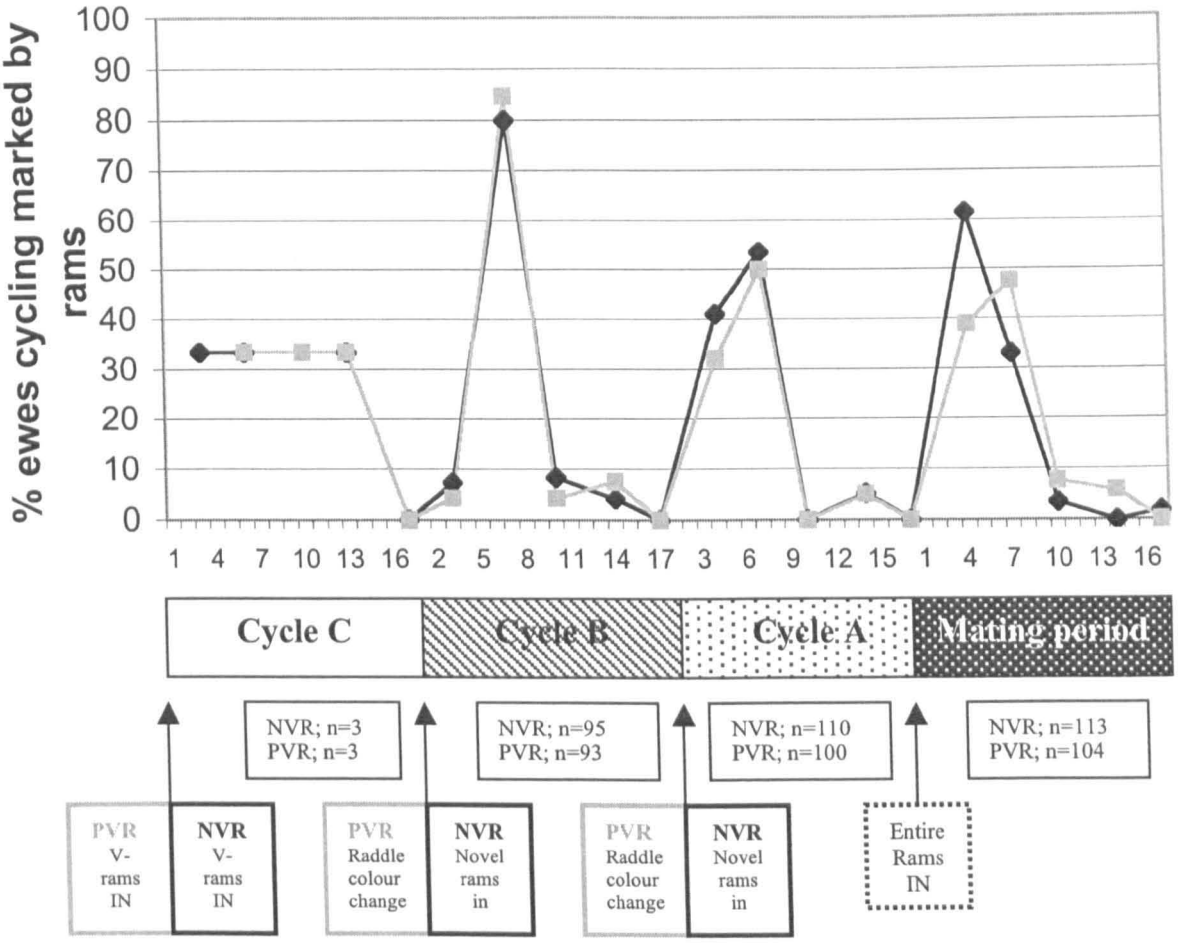


Figure 4.2 Distribution of raddle mark data as a percentage of ewes marked over each 17-day period of permanent ram exposed (PVR, grey square) or novel ram exposed ewes (NVR, black square). Raddle mark data was collected twice weekly during the pre-mating period and daily during the mating period. Therefore the mating data was adjusted to every 3 to 4 days to mimic the frequency of recording during the pre-mating period.

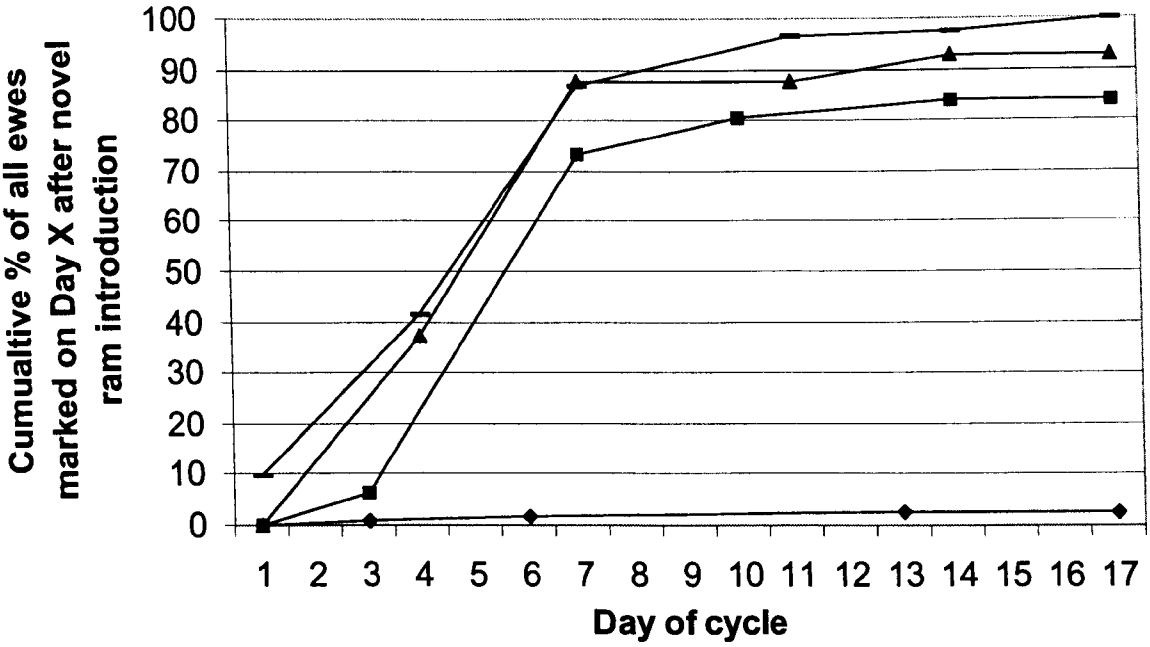


Figure 4.3 Cumulative distribution of raddle mark data for novel ram-exposed ewes over the three oestrous cycles prior to mating (Cycle C; closed diamond, n=3; Cycle B: closed square, n=95; Cycle A: closed triangle, n=110) and at mating (dash, n=113). Raddled vasectomised rams were introduced on Day 0 and novel rams replaced the existing rams on Days 17 and 34, harnessed with a different raddle colour. Entire rams were introduced on Day 50 and raddle mark data from mating is summarised as the number of ewes mated every 3 / 4 days to mimic the pattern obtained by the twice weekly raddle mark checks during the pre-mating period.

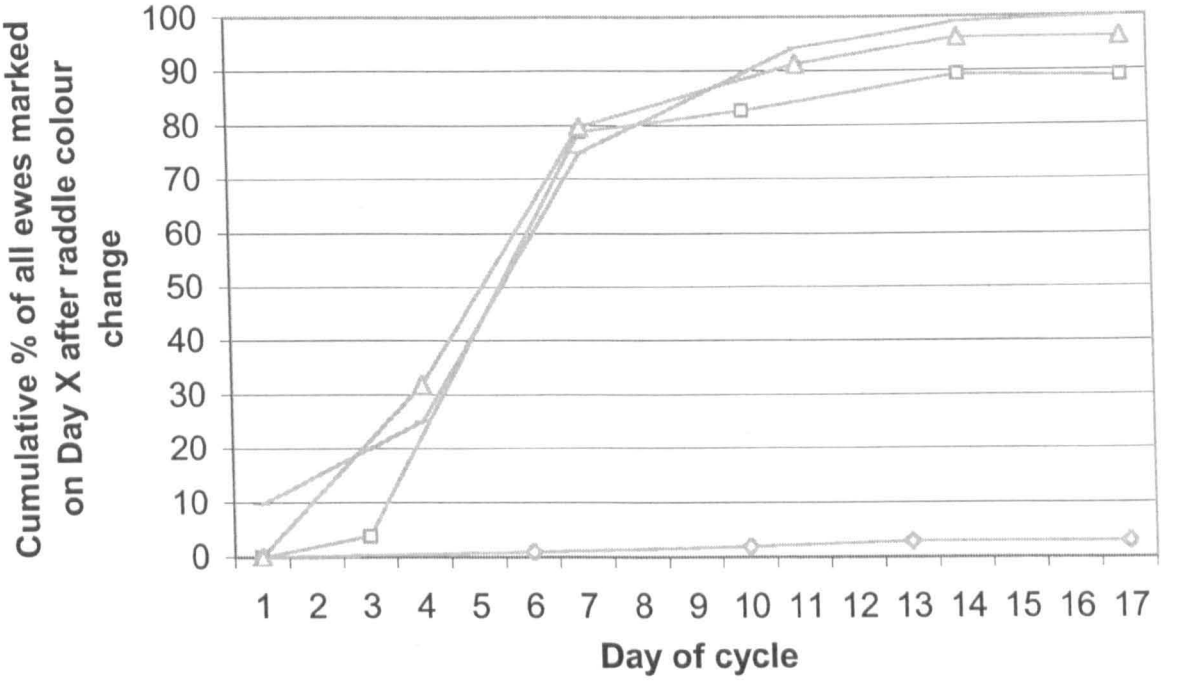


Figure 4.4 Cumulative distribution of raddle mark data for permanently ram exposed ewes over the three oestrous cycles prior to mating (Cycle 1; open diamond, n=3; Cycle 2: open square, n=93; Cycle 3: open triangle, n=100) and at mating (dash, n=104). Raddled vasectomised rams were introduced on Day 0 and raddle colour was changed on Days 17 and 34 and these rams remained with the ewes until mating. Entire rams were introduced on Day 50 and raddle mark data from mating is summarised as the number of ewes mated every 3 / 4 days to mimic the pattern obtained by the twice weekly raddle mark checks during the pre-mating period.

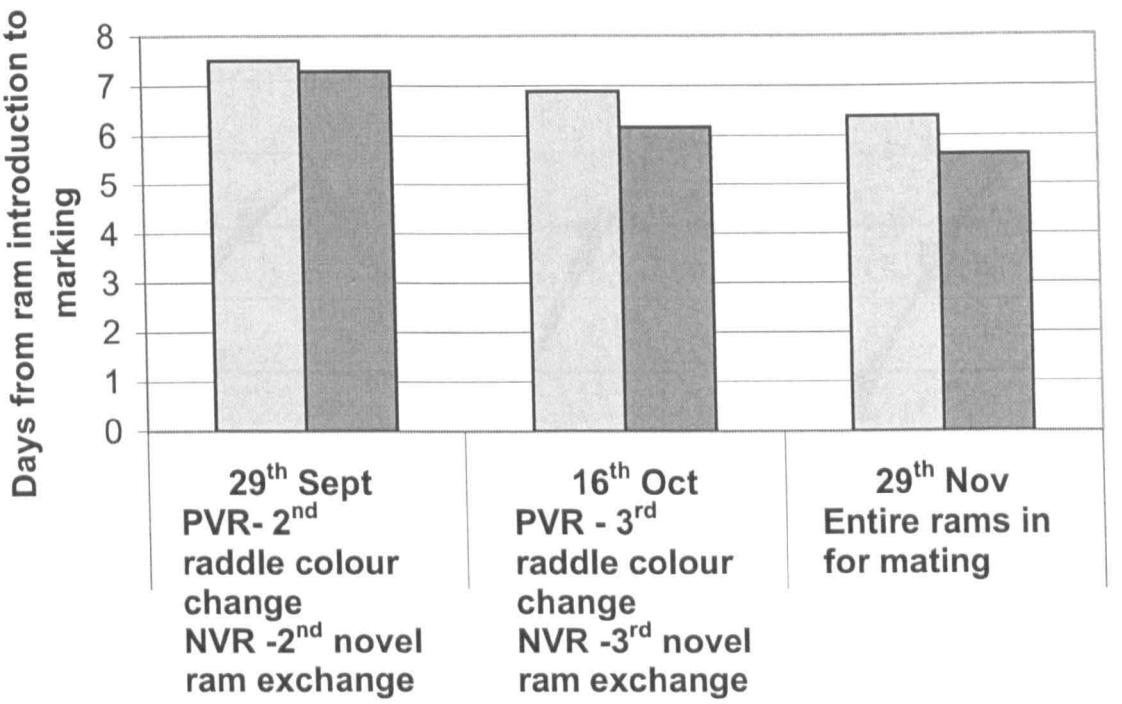


Figure 4.5 Histogram illustrating the mean number of days from the date of raddle colour change (PVR) or novel ram introduction (NVR) to the subsequent date of marking within permanent ram-exposed ewes (PVR, grey solid bars; n=95) and novel ram-exposed (NVR, black hatched bars; n=93). Ewes included in this histogram and data analysis were marked twice prior to mating and at mating. Due to the abnormal distribution of the data, the number of days from ram introduction to subsequent marking by the vasectomised rams or entire rams at mating were compared within treatment by the Wilcoxon Signed Rank test and between treatment by Mann Whitney *U* test. Within PVR ewes there was a significant depression in the days from ram introduction to raddle marking between periods 2 and 3 ($P<0.05$) and between period 3 and mating ($P<0.001$). Within NVR ewes there was similarly a significant depression in between periods 2 and 3 ($P<0.001$) and between period 3 and mating ($P<0.05$). The number of days from ram introduction to raddle marking in NVR ewes was significantly less than in PVR ewes in ram exposure period 3 ($P<0.05$) and at mating ($P<0.01$) but not after ram exposure period 2 ($P>0.05$).

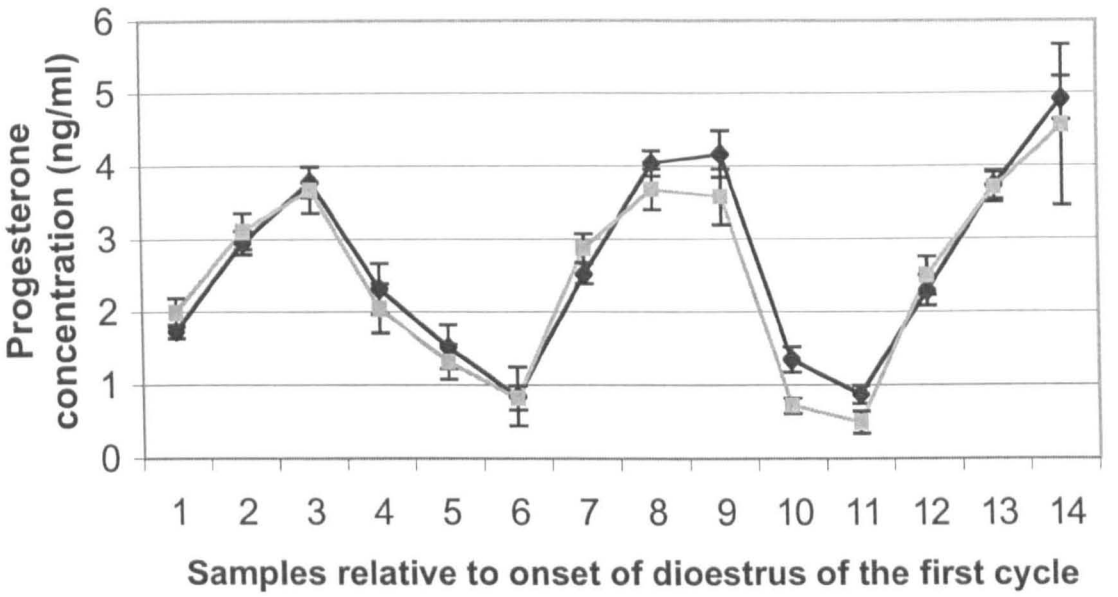


Figure 4.6 Mean (±sem) progesterone concentrations of ewes having three cycles prior to mating which were either permanently exposed to the same vasectomised rams over the pre-mating period (PVR, grey square; n=19) or maintained with vasectomised rams with novel rams introduced every 17 days (NVR, black diamond; n= 21). Blood samples were taken twice weekly from Days –15 to Day 50 of the experiment relative to the day of vasectomised ram introduction (Day 0).

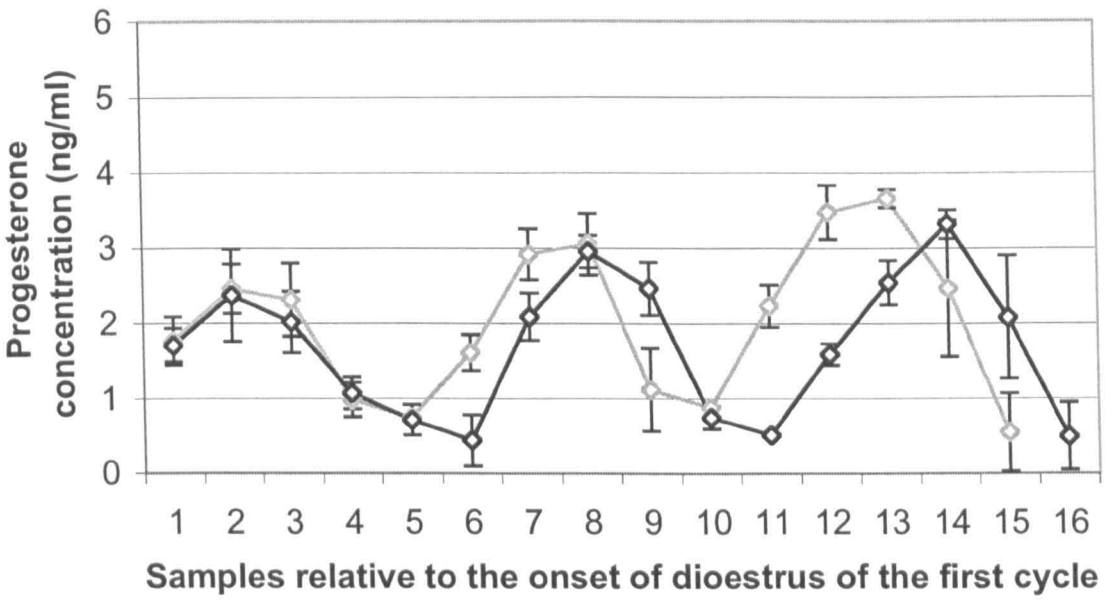


Figure 4.7 Mean (±sem) progesterone concentrations of ewes having three cycles prior to mating that underwent a single 24 hour ram exposure period (SVR, grey open squares, n=8) or were repeatedly exposed to vasectomised ram on Days 0, 17 and 34 of the experiment (RVR, black open diamond, n=5). Blood samples were taken twice weekly from Day –13 to Day 50 of the experiment relative to the day of the first vasectomised ram exposure (Day 0).

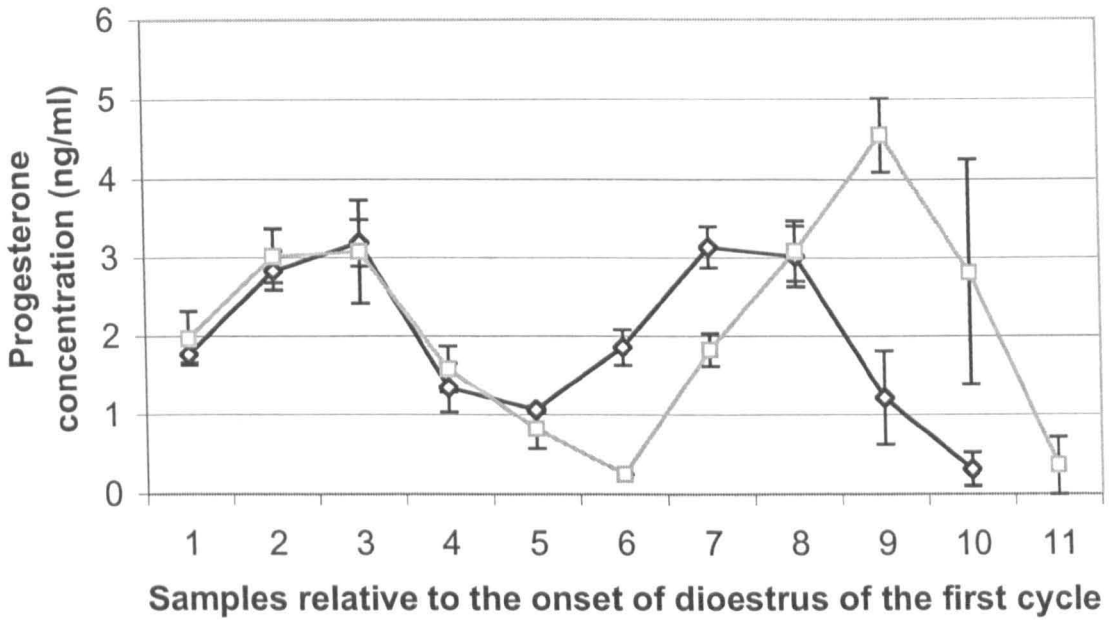


Figure 4.8 Mean (±sem) progesterone concentrations of ewes having two cycles prior to mating which underwent a single 24 hour ram exposure period (SVR, grey open squares, n=9) or were repeatedly exposed to vasectomised rams at on Days 0, 17 and 34 of the experiment (RVR, black open diamonds, n=13). Blood samples were taken twice weekly from Days –13 to Day 50 of the experiment relative to the day of ram introduction (Day 0).

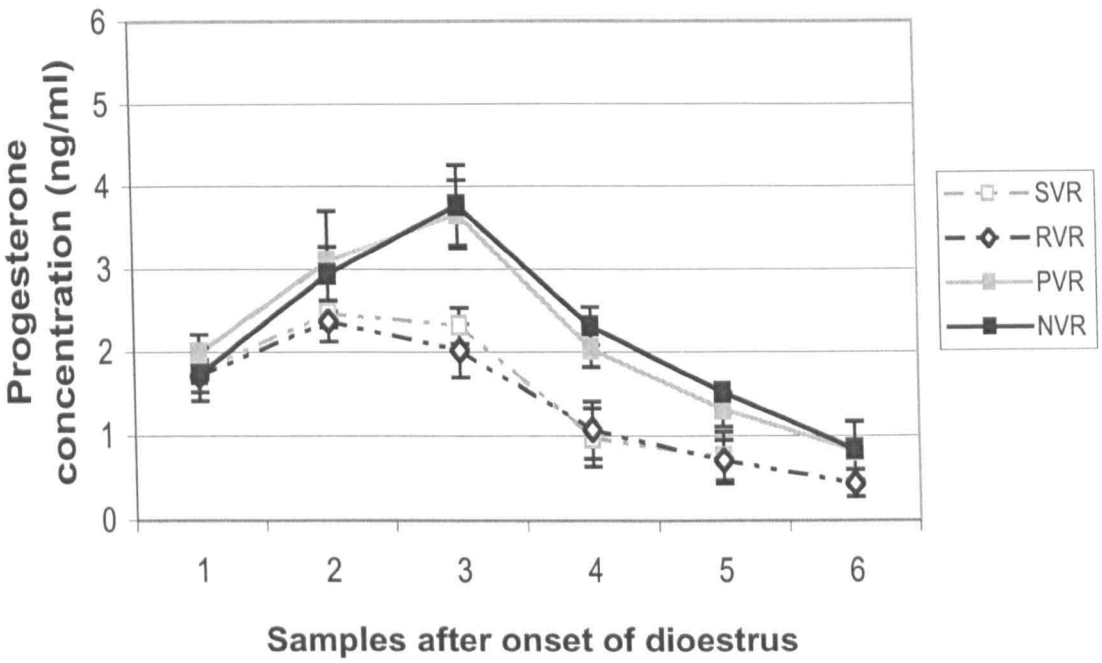


Figure 4.9 Profile of the mean (± sem) concentrations of progesterone during the oestrous cycle, three cycles prior to mating (Cycle C) within the SVR (grey open square; n=8), RVR (black open diamond; n=5), PVR (grey closed square; n=19) and NVR (black closed diamond; n=21) ewes having three oestrous cycles prior to mating. The two groups continuously exposed to rams (PVR and NVR) have comparatively similar progesterone profiles when normalised to the onset of dioestrus with no significant difference in the maximum level of progesterone ($P>0.1$). Similarly ewes isolated from the ram stimulus after a 24 hour ram exposure (RVR and SVR) have similar patterns of progesterone concentrations over the cycle but have markedly different progesterone profiles from the continuous ram exposed ewes and a significantly lower maximum level of progesterone than PVR and NVR ewes ($P<0.01$).

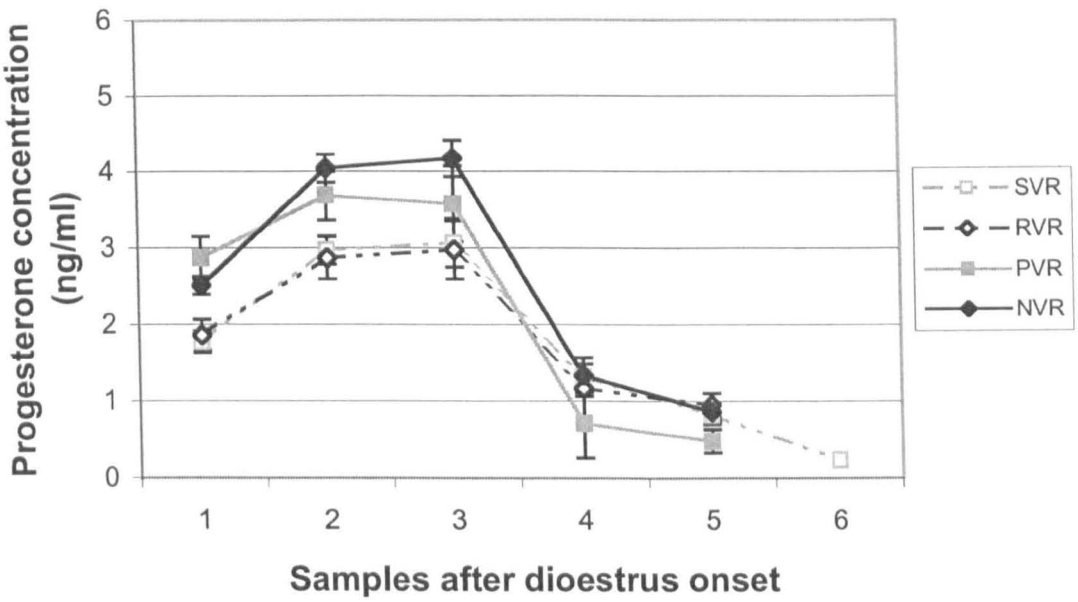


Figure 4.10 Profile of the mean (± sem) concentrations of progesterone during the penultimate oestrous cycle prior to mating (Cycle B) within SVR (grey open square; n=17), RVR (black open diamond; n=18), PVR (grey closed square; n=19) and NVR (black closed diamond; n=21) having at least two oestrous cycles prior to mating. There was no significant difference between NVR and PVR ewes in the maximum concentration of progesterone ($P>0.1$). SVR and RVR ewes again had significantly lower maximum progesterone concentrations than PVR (SVR; $P<0.05$, RVR; $P<0.01$) and NVR ewes (SVR; $P<0.01$, RVR; $P<0.001$) however there continued to be no difference between SVR and RVR ewes in the maximum concentration of progesterone over the cycle ($P>0.1$).

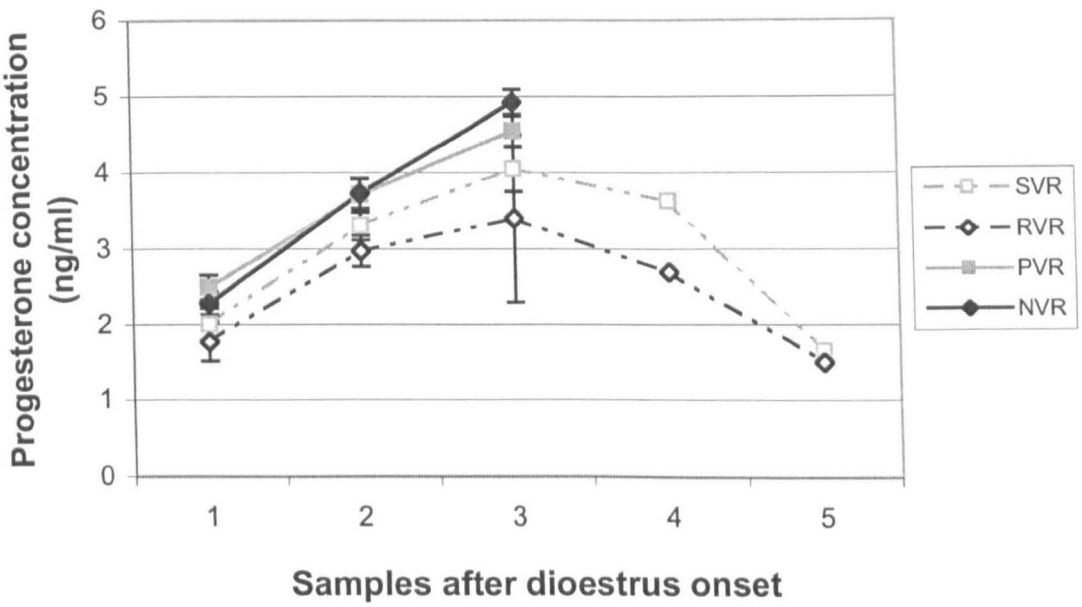


Figure 4.11 Mean (± sem) distribution of the oestrous cycle immediately prior to mating (Cycle A) within all SVR (grey open square; n=20), RVR (black open square; n=20), PVR (grey closed square; n=19) and NVR (black closed square; n=21) ewes. There was no significant difference between NVR, PVR and SVR ewes in the maximum concentration of progesterone ($P>0.1$). However RVR ewes had a significantly lower maximum concentration of progesterone than NVR ewes ($P<0.01$).

4.4.2 MATING AND LAMBING DATA (TABLE 4.2)

The median time of mating occurred earlier in NVR and SVR ewes though this difference was only significant when the median time of mating of NVR and PVR ewes was compared (Table 4.2; $P<0.01$). Significantly more SVR and RVR ewes were mated on Day 1 after entire ram introduction (Table 4.2; $P<0.05$) however this was not sustained after Day 1. In terms of the spread of mating shown in Figure 4.12 the distributions of mating of the four groups were significantly different (Levene's test; $P<0.01$). When considered individually, NVR and PVR ewes had significantly less variance around the median time of mating than SVR ewes ($P<0.001$), indicating a significant compaction and greater synchrony of the mating period within the NVR and PVR ewes. Furthermore both PVR and NVR ewes tended to have less variance around the median time of mating than RVR ewes ($P<0.1$) again indicating improved synchrony of mating. However there was no difference between PVR and NVR ewes or between SVR and RVR ewes in the variance around the median time of mating. This suggests that the distributions of the treatments within the intermittent (SVR versus RVR) and continuous ram exposed ewes (PVR versus NVR ewes) were relatively comparable and that the visual differences in the cumulative distributions of mating (Figure 4.12) were predominantly driven an earlier median time of mating within the SVR and NVR ewes.

Within those ewes lambing to first service, NVR, SVR and PVR ewes all had a significantly earlier median date of lambing than RVR ewes ($P<0.01$). The spread of lambing was significantly different when all groups were considered as illustrated in Figure 4.13 (Levene's test; $P<0.01$). When considering the distribution of the lambing period, NVR and PVR ewes had significantly less variance around the median time of lambing than both SVR and RVR ewes ($P<0.05$) indicating that the synchrony observed at mating continued through to lambing. However in contrast to at mating, NVR ewes had significantly less variance around the median time of lambing than PVR ewes ($P<0.05$) however there remained no significant difference between RVR and SVR ewes.

RVR ewes had a lower mean litter size than SVR, PVR and NVR ewes (1.75 versus 2.05, 1.92, 1.90; RVR, SVR, PVR and NVR ewes respectively). A chi square test showed that this was due to more RVR ewes having single lambs (Table 4.2; $P<0.05$).

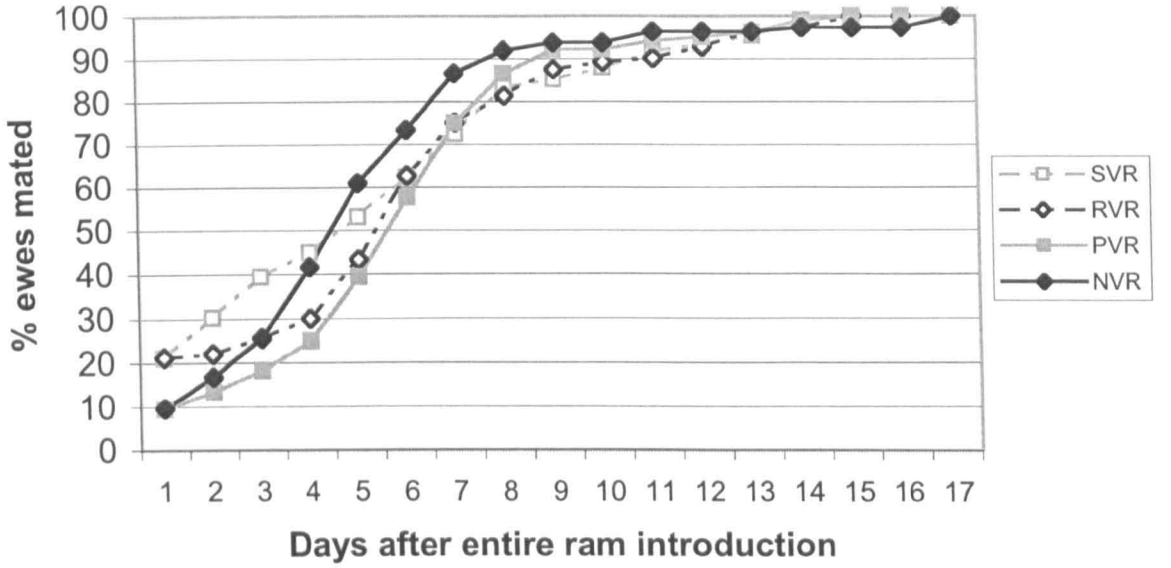
Figure 4.14 shows the litter size relative to the distribution of mating of ewes that lambed to the first service.

There was no significant difference between treatment groups in the numbers of ewes lambing to subsequent services or that were barren, culled or died during the experiment (Table 4.2).

Table 4.2 Effect of different duration and frequency of exposure to vasectomised rams on the time and distribution of mating (days after ram introduction), lambing (days after the onset of lambing within each group) and fertility. Within rows, significant differences are indicated by different superscripts (At least $P<0.05$).

	SVR	RVR	PVR	NVR
Number of ewes	109	113	104	113
Median time from entire ram introduction to mating Days (Interquartile range)	5.0ab (2-8)	6.0 ab (3-7)	6.0b (4.8-7.3)	5.0a (3-7)
Total numbers of ewes bred by: (%)				
Day 1	23a (21)	24a (21)	10b (10)	11b (10)
Day 7	79 (72)	85 (75)	78 (75)	98 (87)
Day 14	108 (99)	110 (97)	103 (99)	110 (97)
Day 17	109 (100)	113 (100)	104 (100)	113 (100)
Within ewes lambing to first service: (%)	96 (88)	100 (88)	92 (88)	97 (86)
Median number of days from ram introduction to lambing Days (Interquartile range)	151a (148-154)	154b (150.8 – 156)	152a (151-154)	151a (150-153)
Total number of ewes lambed by Day X after the onset of lambing of that group: (%)				
1	2 (2)	2 (2)	1 (1)	5 (5)
7	26a (27)	42b (42)	22a (24)	52b (54)
14	78 (81)	92 (92)	85 (92)	94 (97)
17	91 (95)	98 (98)	90 (98)	97 (100)
21	96 (100)	100 (100)	92 (100)	97 (100)
Ewes lambing to first service having: (%)				
1 lamb	11a (11)	26b (26)	14ab (15)	19ab (20)
2 lambs	69 (72)	69 (69)	71 (77)	68 (70)
> 2 lambs	16a (17)	4b (4)	7ab (7)	9ab (9)
Mean litter size (± sem)	2.05 ± 0.05	1.75 ± 0.05	1.93 ± 0.05	1.90 ± 0.05
Ewes lambing to subsequent services (%)	5 (5)	7 (6)	4 (4)	10 (8)
Ewes that were barren, aborted or died (%)	8 (7)	6 (5)	8 (8)	6 (5)

Cumulative distribution of mating



Daily distribution of mating

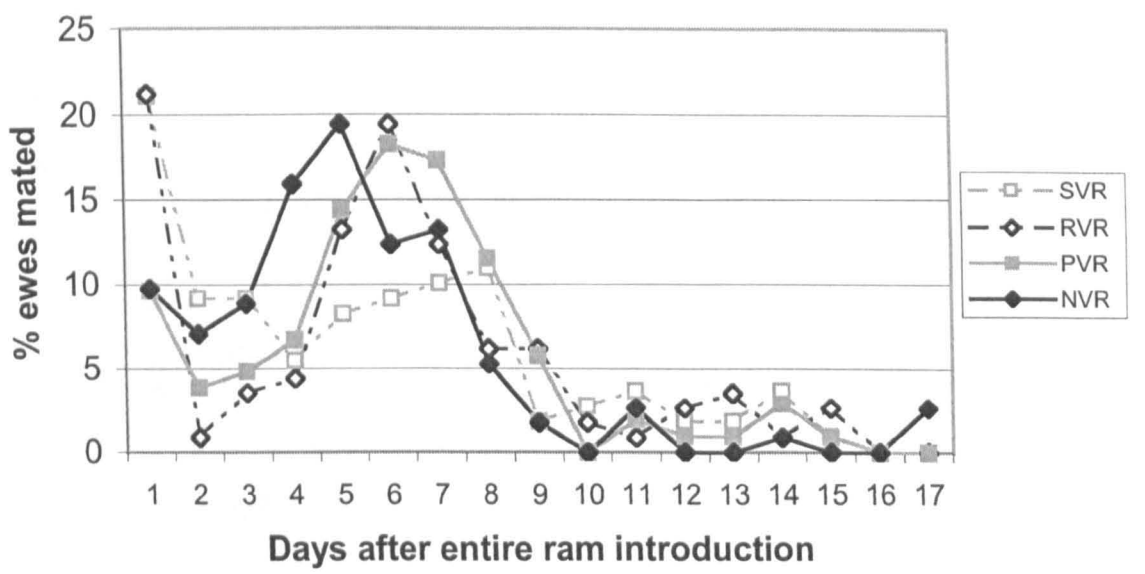
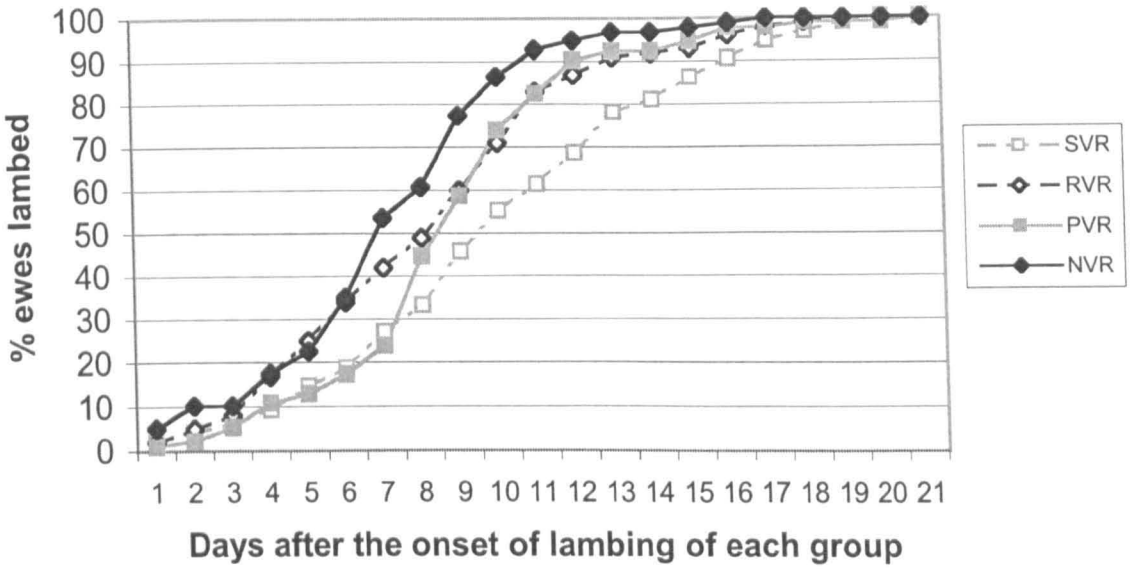


Figure 4.12 Cumulative and daily percentage distributions of mating of novel ram exposed ewes (NVR, black diamond, solid line; n=113), permanent ram exposed ewes (PVR, grey square, solid line; n=104) repeated ram exposed (RVR, open black diamond, dashed line; n=113) and single ram exposed ewes (SVR, open grey square, dashed line; n=109).

Cumulative distribution of lambing



Daily distribution of lambing

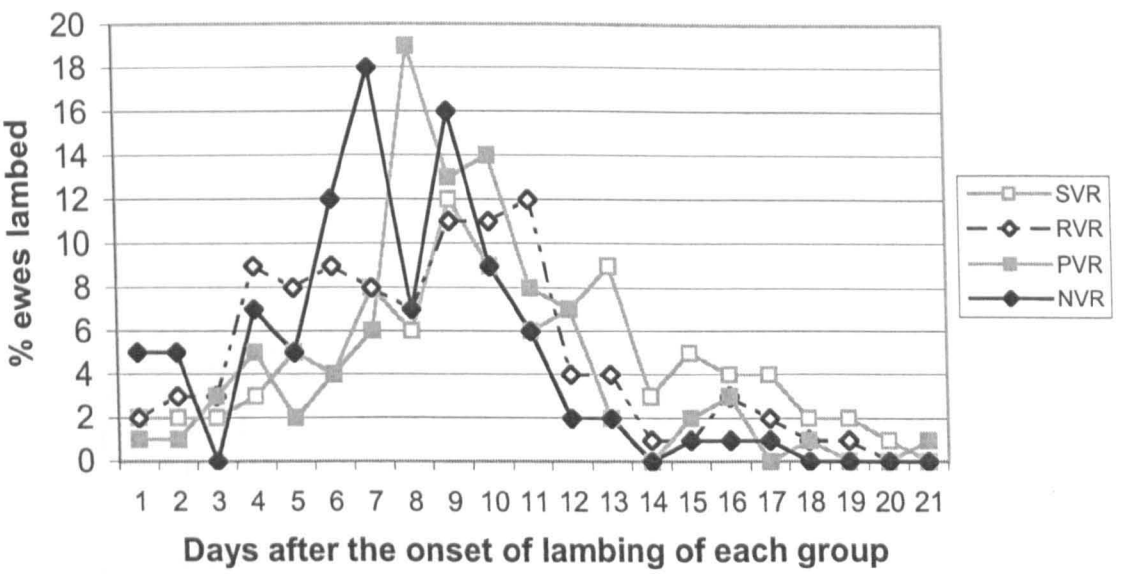


Figure 4.13 Cumulative and daily percentage distributions of lambing of novel ram exposed ewes novel ram exposed ewes (NVR, black diamond, solid line; n=97), permanent ram exposed ewes (PVR, grey square, solid line; n=92) repeated ram exposed (RVR, open black diamond, dashed line; n=100) and single ram exposed ewes (SVR, open grey square, dashed line; n=96). The onset of lambing in each group was 142, 146, 144 and 145 days after entire ram introduction for SVR, RVR, PVR and NVR ewes respectively.

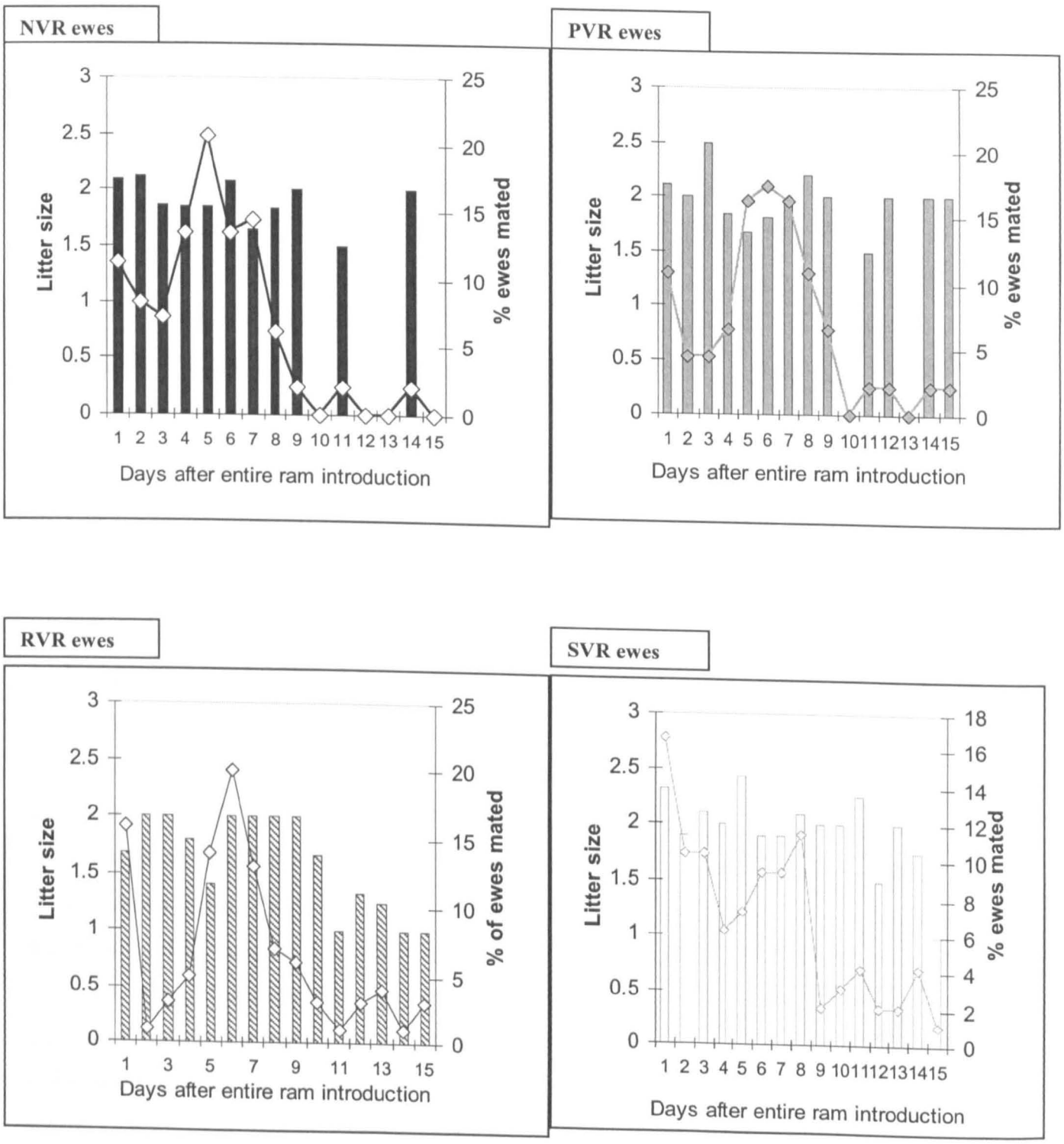


Figure 4.14 Comparisons of litter size (bars) relative to the daily distribution of mating within those ewes that lambed to the first service (line) for novel ram exposed ewes novel ram exposed ewes (NVR; n=97), permanent ram exposed ewes (PVR; n=92) repeated ram exposed (RVR; n=100) and single ram exposed ewes (SVR; n=96).

4.5 DISCUSSION

Ewes maintained in continuous presence of rams from the transition into the core of the breeding season had an earlier onset of cyclic activity and were mated over a more compact period than ewes exposed intermittently to rams. Continued ram presence is typically deemed necessary for maintenance of an LH response to the ram effect (Oldham and Pearce, 1983; Pearce and Oldham, 1984; Murtagh *et al.*, 1984b). Therefore though within Chapter 3, I showed conclusively that a 24-hour ram exposure is effective in inducing ovulation in a proportion of ewes, this study shows a heightened response when ewes are maintained continuously with rams. This enhanced response to the ram is expressed in two ways; firstly by the greater number of ewes having three oestrous cycles prior to mating and secondly by the higher maximum concentrations of progesterone during the pre-mating period.

The earlier median onset of cyclic activity in the continuously ram-exposed ewes was driven by a greater proportion of ewes having the onset of the first oestrous cycle of the breeding season within 17 days of vasectomised ram introduction. In contrast a markedly smaller proportion of the single and repeated ram exposed ewes showed a similar initiation of cyclic activity in this 17-day period. The mule ewe is capable of responding to the ram effect during late anoestrus with an increase in LH concentrations (Stansfield *et al.*, 1987; Al-Mauly *et al.*, 1991). Based on observations in anoestrous ewes, removal of the ram is likely to be associated with a rapid depletion in LH concentrations and a reversion to an anoestrous endocrine state (Signoret *et al.*, 1982; Oldham and Pearce, 1983). However in this study ewes were introduced to rams during the transition into the breeding season where fluctuations in LH can be sufficient to induce the onset of the first oestrous cycle of the breeding season (Karsch *et al.*, 1984). Therefore the repeated and single ram exposed ewes that did begin cyclic activity within one cycle length of the initial ram exposure period were likely to be close to the natural onset of cyclic activity. I propose that the short-term ram induced increase in LH was sufficient to stimulate the onset of the first oestrous cycle of the breeding season within a proportion of short-term ram exposed ewes, irrespective of the removal of the ram stimulus. In contrast the maintenance of ram presence in the continuously ram exposed ewes provided ewes with continuous stimuli to advance and compact the onset of cyclic activity. The concentration of behavioural oestrus in the continuously ram exposed ewes between 24 and 25 days

after initial vasectomised ram introduction (Figure 4.2) indicates a high occurrence of either short cycles (6-7 days; Martin *et al.*, 1986) or delayed ovulation (Ungerfeld *et al.*, 2002). The occurrence of such phenomena is also likely in the repeated and single ram exposed ewes, however the removal of the ram may have resulted in a reversion to an anoestrous state until the natural onset of cyclic activity or the subsequent ram exposure period in the repeated ram exposed ewes.

Continuously ram exposed ewes not only had a higher percentage of ewes having three oestrous cycles prior to mating but also comparatively higher maximum progesterone concentrations over the three oestrous cycles prior to mating. I have outlined the significance of continued ram presence in stimulating the onset of reproductive activity however I propose that it may also modulate progesterone production during the luteal phase. Gonadotrophic support during both the follicular phase and early luteal phase is critical to the subsequent lifespan and progesterone production of the corpus luteum (Review; Garverick *et al.*, 1992) and sustained ram presence is likely to have maintained the ram induced increase in LH concentrations (Martin *et al.* 1986). During the follicular phase, both FSH and LH are involved in the preparation of follicles for the process of luteinsiation (Garverick *et al.*, 1992) and inadequate gonadotrophic support can restrict the capacity of these cells to secrete progesterone (Garverick and Smith, 1986). During the luteal phase, elimination of LH using a GnRH antagonist caused a depression in maximum progesterone production from the corpus luteum (Peters *et al.*, 1994). LH release during the early luteal phase is particularly critical and restriction resulted in formation of a corpus luteum with a normal lifespan but suppressed maximum progesterone concentrations (Peters *et al.*, 1994). Therefore I hypothesise that the persistently enhanced progesterone production within ewes maintained continuously with rams was a consequence of enhanced luteal development and function.

Ewes exposed to novel rams every 17 days were mated had a one-day advance in the median date of mating than ewes maintained permanently with the same rams. The percentage frequency of cyclic ewes marked by the vasectomised rams over pre-mating period indicates that the divergence between the permanent and novel ram exposed ewes occurred during cycle prior to mating and at mating. This evidence is supported by the significant depression in the number of days from the date of novel

ram introduction to the subsequent date of marking over time in the novel ram exposed ewes. It is of particular significance that this is also accompanied by a divergence in the median onset date of dioestrus. At the onset of the breeding season, the permanent and novel ram exposed ewes had the same median dioestrous onset date compared to at mating where the novel ram exposed ewes were mated significantly earlier (1 day) than the permanent ram exposed ewes. This concept of a shift in the location rather than the distribution of oestrus of the novel ram exposed ewes is also evident in the changes in the cumulative distribution of raddle mark data. I propose that the divergence between these continuously ram exposed ewes was due to the introduction of novel rams every 17 days. I hypothesise that the absence of any significant differences in cycle length between the blood-sampled subsets of ewes is in part a reflection of the twice-weekly blood-sampling regime. The development of a one-day advance in the median dioestrous onset date over a 50-day period would require daily blood samples to detect the subtle and progressive changes in dioestrous onset indicated by the raddle mark data.

I hypothesised that a single short term ram exposure period would be sufficient to affect the distribution of the onset of the breeding season and at mating. Furthermore I aimed to determine any benefit of the subsequent ram exposures in the repeated ram exposed ewes on the synchrony at mating. The distributions obtained from a single and repeated ram exposed ewes both show an initial peak of oestrus on Day 1 after entire ram introduction. However the repeated ram exposed ewes have a second distinct peak of oestrus at Days 5-7 after entire ram introduction compared to a more protracted distribution in the single ram exposed ewes. This divergence is shown statistically by the significant differences in their synchrony scores. The synchrony score determines the absolute difference in dioestrous onset dates between all ewes within a group. Therefore the protraction of mating within the single ram exposed ewes caused a higher synchrony score, indicating that the ewes were less synchronous at mating than the repeated ram exposed ewes. The concept of synchrony is difficult to define due to natural variation in cycle length and criticism of the methods used to measure synchrony (Schank, 2000; 2001). This controversy is evident in the absence of a significant difference in the median and variance around the median date of mating between the two groups of ewes. However I propose that the calculation and comparison of groups by synchrony score is a valid and relevant method of examining

the mating distribution as it considers both the shape and location and shows statistically the visual differences in the distributions of the groups at mating.

The single ram exposed ewes had a peak of mating over the first 3-4 days after entire ram introduction. I propose that this early concentration of mating is a consequence of the large proportion (40%) of ewes ovulating in response to the first ram exposure period. The proportion of ewes ovulating after a 24-hour ram exposure is higher than that observed by Signoret *et al.,* (19%; 1982) and in repeated ram exposed ewes both in this study (25%) and in Chapter 3 (21%). Though evidence of a female-to-female effect in sheep is divisive, studies that have indicated a female-to-female effect (Zarco *et al.,* 1995) used a high percentage of oestrous females (50%) to anovular females. Therefore in the absence of subsequent ram exposures the ewes initially stimulated by the 24-hour ram exposure may have had a residual effect on the onset of cyclic activity in the remaining anovular ewes thus resulting in the concentration of mating within the first 3-4 days of entire ram introduction.

Repeated ram exposed ewes had a significantly lower litter size than the single ram exposed ewes driven by proportionately more single and less multiple births. The two groups of ewes were maintained at the same location but on different pastures to maintain single ram exposed ewes in isolation from ram contact. Therefore I cannot discount the possibility of this being a reflection of differences in pre-mating nutrition, which is critical factor in determining ovulation rate (Review, Gunn, 1982). Furthermore the proportion of the repeated ram exposed ewes mated at the end of the mating period (14%) had predominantly single lambs. Semen quality has been shown to decline over time relative to the time of joining with ewes (Raadsma and Edey, 1984). Therefore the presence of two peaks of activity followed by a residual number of ewes mated may have resulted in a dilution effect on the quality of the semen in the late-mated ewes. Semen quality is correlated with the number of lambs born per ewe (Hulet *et al.,* 1965) therefore this could be the origin of the reduced litter size in these late mated ewes. However the observation that these ewes do not account for all the single births in addition to the observed trend towards a reduced litter size in ram exposed ewes in Chapter 3 indicates that this observation may have been due to an effect of the repeated ram exposures during the pre-mating period.

I propose two possible mechanisms that could have resulted in a ram-induced depression in litter size. Repeated ram exposed ewes had a distinct bimodal distribution of mating activity on Day 1 and Days 5-7 after entire ram introduction. The raddle mark data indicates that a proportion of ewes were in oestrus during the second and third ram exposure periods. Continuous ram presence during pro-oestrus and oestrus significantly advances the LH surge and ovulation (Lindsay *et al.*, 1975). Therefore as the period available for folliculogenesis is a critical determinant of ovulation rate (Scaramuzzi *et al.*, 1993) the introduction of entire rams and associated LH response may have stimulated the LH surge at a time when a limited number of follicles were developmentally capable of responding. I propose that the absence of a depression in litter size in the large proportion of single ram exposed ewes mated at this time is a consequence of a cumulative effect of the repeated ram exposures during the pre-mating period on ewes in or approaching oestrus at the time of ram exposure.

Ewes mated during the second peak of oestrous activity would have been in their luteal phase when entire rams were introduced. Evans *et al.*, (2004) and Hawken *et al.*, (2005) showed that ram exposure towards the end of artificial progestagen treatment was associated with an earlier oestrus and ovulation and a significantly lower litter size. This reduced litter size was due to a depression in ovulation rate that was driven by proportionately more single ovulations in the ram-exposed ewes (Hawken *et al.*, 2005). Similarly in this study the lower litter size in the repeated ram exposed ewes was driven by these ewes having more single lambs than the single ram exposed ewes. Ewes mated at the second peak of mating had a numerically lower mean litter size specifically when compared to single ram exposed ewes mated at the same time relative to entire ram introduction. It is interesting to note that though the novel and permanent ram exposed ewes did not have a significant depression in litter size compared to single ram exposed ewes they also exhibit a comparatively low litter size at this second peak of mating activity.

In summary, continuous exposure of ewes to rams during the transition into the breeding season stimulates the onset of cyclic activity in a greater proportion of ewes and yields a more compact distribution of mating when compared to ewes exposed intermittently to rams. Within the permanently ram exposed ewes, periodic introduction of novel rams caused a movement in the location but not distribution of

the oestrous activity however the mechanism driving this difference is not clear. A single ram exposure period is sufficient to affect the timing and distribution of the onset of cyclic activity within a proportion of ewes. The variation in the patterns of mating observed in this study infers the potential for tailoring the pattern of mating towards the most desirable distribution of lambing for an individual farmer.

PAGE

NUMBERING

AS ORIGINAL

Zarco L, Stabenfeldt GH, Quirke JF, Kindahl H and Bradford GE (1988). Release of prostaglandin F-2 alpha and the timing of vents associated with luteolysis in ewes with oestrous cycles of different lengths. *Journal of Reproduction and Fertility*; 83:517-526

Zarco L, Rodriguez EF, Angulo MRB, Valencia J (1995). Female to female stimulation of ovarian activity in the ewe. *Animal Reproduction Science*; 39:251-258

Ziecek AJ, Bodek G, Ciereszko R, Stepien A, Kotwica G (2001). Involvement of gonadotrophins in induction of luteolysis in pigs. *Reproductive Biology*; 1:33-50

Ziecek AJ, Bodek G, Blitek A, Kaczmarek A, Waclawik N (2004). Nongonadal LH receptors, their involvement in female reproductive function and a new applicable approach. *The Veterinary Journal*; 169(1): 75-84.

Zuniga O, Forcada F, Abecia JA (2002). The effect if melatonin implants on the response to the male effect and on the subsequent cyclicity of Rasa Aragonesa ewes implanted in April. *Animal Reproduction Science*; 72(3-4): 165-174

6. INVESTIGATION INTO THE EFFECT OF RAM INTRODUCTION DURING THE BREEDING SEASON ON THE ENDOCRINE RESPONSES OF RANDOMLY CYCLING MAIDEN EWES WITH OR WITHOUT PRIOR EXPERIENCE WITH THE RAM DURING ANOESTRUS.

6.1 ABSTRACT

The ram effect is not limited to the anoestrous period and the absence of a significant effect of prior experience with the ram on the subsequent endocrine response observed in Chapter 5 may not be representative of maiden ewes introduced to the ram during the breeding season. Therefore a subset of the maiden ewes that were either preconditioned during anoestrus as outlined in Chapter 5 (RE; n=6) or isolated from ram contact (RN; n=6) were introduced to rams during the breeding season (October) midway through a frequent blood-sampling regime. Both RE and RNA ewes had a significant increase in mean ($P<0.01$) and basal ($P<0.05$) LH concentrations and an increase in LH pulse frequency (RE, $P<0.05$; RN, $P<0.1$) in response to ram introduction, however there was no effect on LH pulse amplitude. There was no significant effect of prior experience with the ram on any parameters of the LH response. Therefore data was pooled and regrouped according to stage of the oestrous cycle at the time of ram introduction. The presence of only one ewe in the follicular and mid luteal phase prevented analysis of ewes in their early or late luteal phase. Ewes in the early (EL) and late luteal (LL) phase responded to ram introduction with an increase in mean (EL, $P<0.1$; LL, $P<0.01$) and basal (EL, $P<0.05$; LL, $P<0.05$) LH concentrations and LH pulse frequency (EL, $P<0.1$; LL, $P<0.05$) however there was no effect on LH pulse amplitude. The capacity of ewes irrespective of stage of the oestrous cycle to respond to ram introduction with a characteristic LH response reiterates the potency of socio-sexual cues of the ram in mediating physiological changes in the reproductive activity of the ewe.

6.2 INTRODUCTION

The ability of the ram to stimulate LH secretion (Pearce and Oldham, 1983) and influence the reproductive state of ewes is not restricted to the anoestrous period (Lindsay *et al.*, 1975). Ram introduction to ewes whilst under the influence of an artificial progestagen induces an increase in LH concentrations in ewes treated with the progestagen during both anoestrus (Martin *et al.*, 1983b) and the breeding season (Pearce and Oldham 1983, Evans *et al.*, 2004). Similarly the continuous presence of rams during an artificially induced or natural follicular phase advanced the onset and reduced the duration of oestrus (natural; Parsons and Hunter, 1967; Fletcher and Lindsay, 1971, artificial; Maxwell, 1986; Romano *et al.*, 2000; 2001).

Therefore it is apparent that modification of the balance of gonadotrophin secretion during the different stages of the oestrous cycle within artificially synchronised ewes can have residual effects on follicle development and the subsequent sequence of endocrine events. This raises the question as to what effect ram introduction may have at differing stages of a naturally occurring oestrous cycle? Chapter 5 showed a significant effect of prior experience with the ram during anoestrus on the behavioural interactions between the ewes and rams however there was no significant effect on the endocrine response to ram introduction. However when introduced to rams at a synchronised oestrus, Gelez *et al.*, (2004a) identified a shorter duration of the LH surge in young sexually naïve maiden ewes compared to sexually experienced adult ewes. Consequently there may be a differential effect of prior experience with the ram on the LH response in maiden ewes when introduced to rams during the breeding season that is no evident during anoestrus.

Therefore the aim of this experiment was to investigate the endocrine response of cyclic maiden ewes to ram introduction at different stages of the oestrous cycle and any interaction with prior experience with the ram during anoestrus. Specifically I will look at the differences in LH pulsatility, pulse amplitude, circulating and basal concentrations of LH in response to ram introduction and the number and timing of ewes having an LH surge. I also aim to detect, where possible, any effects of ram introduction on oestrous cycle length before, during and after ram introduction.

6.3 MATERIALS AND METHODS

6.3.1 ANIMALS AND EXPERIMENTAL PROCEDURES

A subset of the ram experienced and ram naïve maiden mule (Swaledale x Bluefaced/Border Leicester) ewes from Chapter 5 were drafted off prior to ram introduction to the main group of ram experienced and ram naïve ewes in Experiment 1 of Chapter 5. This subset of ewes (ram experienced, RE: n=6; ram naïve RN: n=6) were housed in mixed groups of 3 ewes per pen (2m x 1.8m) and maintained under an artificially controlled natural lighting regimen. As in Chapter 5, vasectomised rams were used throughout this experiment but will be referred to as rams for the purpose of simplicity.

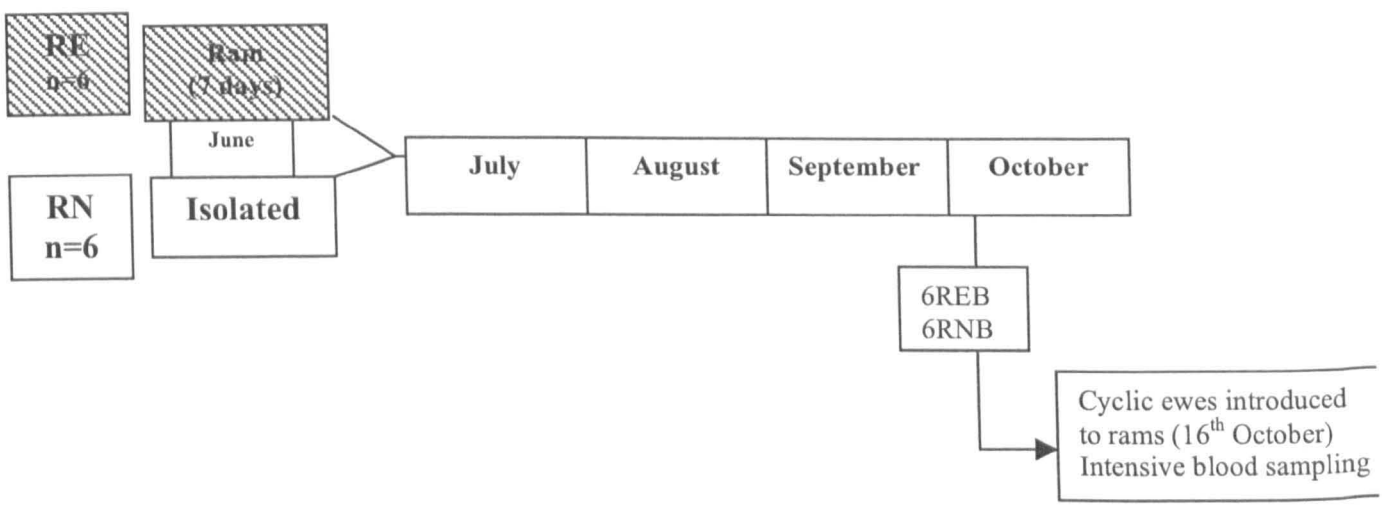


Figure 6.1 On Day 0 of the experiment (16th October), rams (n=4) were introduced midway through the serial bleed procedure (1pm) outlined below and remained with the ewes for the remaining duration of the experiment.

6.3.2 CANNULATION PROCEDURE AND BLOOD PROCESSING

The cannulation procedure, maintenance of the cannulae and processing of the blood samples were carried out as in Chapter 5.3.4.2 and 5.3.5 respectively.

6.3.3 BLOOD COLLECTION

On the day of ram introduction (Day 0), frequent blood samples (5ml) were collected via a jugular cannula, every 12 minutes during the 6 hours before and 6 hours after ram introduction. On Day 1 after ram introduction, blood samples were taken every 2 hours

via the jugular cannula from 19 to 43 hours after initial ram introduction to attempt to detect the LH surge.

Blood samples (5ml) were collected by jugular venepuncture (Vacutainer, Becton-Dickinson Limited, Coventry) or via the cannula for progesterone in order to establish that the ewes were cyclic prior to ram introduction, to determine their stage of cycle at ram introduction and to monitor their subsequent progesterone profile. As in Chapter 5, blood samples were taken twice weekly for 2 weeks prior to ram introduction with the frequency increased to daily during Days 3 to 6 after ram introduction. On Day 6 the cannulae were removed and the frequency of samples rescheduled to twice weekly via jugular venepuncture for three weeks after ram introduction.

6.3.4 IMMUNOASSAY

Plasma progesterone concentrations were analysed in duplicate using a commercial enzyme linked immunoassay (ELISA) kit (Ridgeway Science Ltd, Gloucester, UK) as outlined in Chapter 3.3.4. Mean intra-assay and inter-assay coefficients of variation for low (1.85ng/ml), medium (3.02ng/ml) and high (7.43ng/ml) plasma samples were 7.6% and 11.4%, 2.7% and 10.5% and 7.4 and 8.0% respectively. The sensitivity of the assay was 0.23ng/ml.

Serum LH concentrations were determined using a previously validated double antibody radioimmunoassay as outlined in Chapter 5.3.6. Mean intra-assay and inter-assay coefficients of variation for low (0.28ng/ml), medium (1.53ng/ml) and high (3.47ng/ml) plasma samples were 7.1% and 16.2%, 7.8% and 8.2% and 8.4% and 18.6% respectively. The sensitivity of the assay was 0.1ng/ml.

6.3.5 DATA ANALYSIS

Blood samples collected during the two weeks prior to ram introduction were used to formulate a progesterone profile for each ewe. The stage of oestrous cycle at ram introduction was determined from the progesterone concentration at the beginning of the serial bleed on the day of ram introduction. A sample from each ewe taken at the end of the serial sampling period was also analysed to determine progesterone concentrations after ram introduction. Using the progesterone profiles, oestrous cycle

length was calculated as the number of days between the nadir points of two successive oestrous cycles.

Serum LH concentrations were plotted out for individual ewes over the duration of the 12 hour blood sampling period. As the secretion of LH was pulsatile, the frequency of pulses was analysed using the Munro algorithm, which is a modified version of the Pulsar algorithm (Wachter and Merriam, 1982). The parameters for the Munro analysis were the same as those outlined in Chapter 5.3.7. The programme calculated the nadir and amplitude for each pulse and the mean nadir and pulse amplitude.

Data for LH pulse frequency, LH pulse amplitude, circulating and basal LH concentrations before and after ram introduction were subject to repeated measures ANOVA in Genstat 5 (for Windows Second Edition) primarily to assess the effects of ram experience, time relative to ram introduction (before or after) and any time/ram experience interactions. Basal and circulating LH concentrations required log 10 transformation prior to analysis due to the data being skewed. On account of the confounding factor of stage of oestrous cycle on the capacity of a ewe to have an LH surge this aspect of LH secretion was not considered relative to prior experience with the ram.

Due to the absence of an effect of ram experience on any parameters of the LH response to ram introduction, data for ram experienced and ram naïve ewes were pooled and ewes re-categorized by stage of oestrous cycle using their progesterone profiles. The LH data (transformed where necessary) and progesterone data before and after ram introduction were analysed by repeated measures ANOVA to assess the effects of stage of oestrous cycle, time relative to ram introduction and any time/ stage interactions. Where a significant effect of ram introduction was detected, data before and after ram introduction was compared by Paired T-test (Minitab, 13.1)

The numbers of ewes detected with an LH surge within the sampling period were recorded for each stage of the oestrous cycle. Occurrence of an LH was determined as outlined in Chapter 5.3.7. The latency to the LH surge, and duration of the surge were recorded where possible.

6.4 RESULTS

All ewes were confirmed as actively cycling (from their progesterone profiles) on the date rams were introduced during the breeding season. Ram introduction caused a significant increase in mean and basal LH concentrations and LH pulse frequency in all ewes, however there was no significant effect on LH pulse amplitude (Table 6.1). There was no significant effect of prior experience with the ram during anoestrus on the endocrine response to ram introduction during the breeding season (mean and basal LH concentrations, LH pulse frequency or LH pulse amplitude, $P>0.1$; Table 6.1).

In the absence of a significant effect of prior experience with the ram, data for ram naïve and experienced ewes were pooled and ewes were re-classified by their stage of oestrous cycle at the time of ram introduction (based on their progesterone profiles). One ewe was classified as in the follicular phase, three ewes in the early luteal phase, one ewe in the mid-luteal phase and six ewes in the late luteal phase. One ewe was on the down slope of the LH surge at the beginning of the frequent sampling period thus was excluded from analysis however the profile for this ewe is presented in Figure 6.6 Due to there being only one representative ewe in the follicular phase and mid luteal phase, statistical analysis was conducted only on ewes in their early or late luteal phase. There was no significant effect of stage of the luteal phase on the endocrine response to ram introduction (Table 6.2; $P>0.1$).

Ewes classed (based on their progesterone profiles) as in their early luteal phase on the day of ram introduction ($n=3$) showed a significant increase in basal LH concentrations ($P<0.05$) and tended to show an increase in mean LH concentrations (Table 6.2; $P<0.1$). After ram introduction there was a numerical increase in the number of LH pulses over the 6-hour sampling period however this again only tended towards significance (Table 6.2; $P\leq0.1$). Furthermore there was no effect of ram introduction on LH pulse amplitude (Table 6.2)

Ewes classed as in their late luteal phase on the day of ram introduction ($n=6$) showed a significant increase in basal and mean concentrations of LH ($P<0.01$ and $P<0.05$ respectively) and the number of LH pulses in the 6-hour sampling period after ram introduction ($P<0.05$; Table 6.2). However there was no significant effect of ram introduction on LH pulse amplitude ($P>0.1$). During the period before and after ram

introduction there was a significant decrease in mean concentrations of progesterone (Table 6.2; $P<0.05$).

Only ewes classed as in their follicular phase or late luteal phase at the time of ram introduction had an LH surge during the blood-sampling period between 19 and 43 hours after ram introduction (follicular phase, 1/1; luteal phase, 4/6). In the ewes in their late luteal phase, the average latency to the LH surge was 34.3 ± 4.8 hours after ram introduction (excluding one ewe where the surge began before the sampling period). The ewe in the follicular phase on the day of ram introduction had the onset of the LH surge prior to the sampling period and LH concentrations returned to basal values at 33 hours after ram introduction. It was not appropriate to determine the maximum concentration of the LH surge, as it was impossible to determine the concentration within those ewes having the peak of the LH surge outside of the sampling period. Only one ewe had the onset and end of the LH surge within the sampling period thus preventing any analysis of LH surge duration relative to the LH response parameters.

Within ewes in their late luteal phase on the day of ram introduction (n=6) the oestrous cycle following ram introduction tended to be shorter in length than the oestrous cycle during which rams were introduced (Figure 6.7; $P\leq0.1$). A similar analysis was not possible with ewes in the early luteal phase (n=3) due to the lack of a nadir point for the cycle prior to and after ram introduction. However within these ewes mean cycle length of the cycle when rams were introduced was 17.67 ± 0.67 days and the mean distribution of cycles is shown in Figure 6.8

The ewe in the follicular phase at the time of ram introduction (n=1) had a decrease in cycle length during the cycle after rams were introduced (13 versus 19 days). The profile for the ewe in the mid luteal phase at the time of ram introduction (n=1) showed that the cycle length of the cycle when rams were introduced was shorter than that of the subsequent oestrous cycle (15 versus 17 days).

Progesterone profiles of oestrous cycles before and after ram introduction categorized by stage of oestrous cycle and relative to their LH response, are presented for individual ewes in Figures 6.2-6.6.

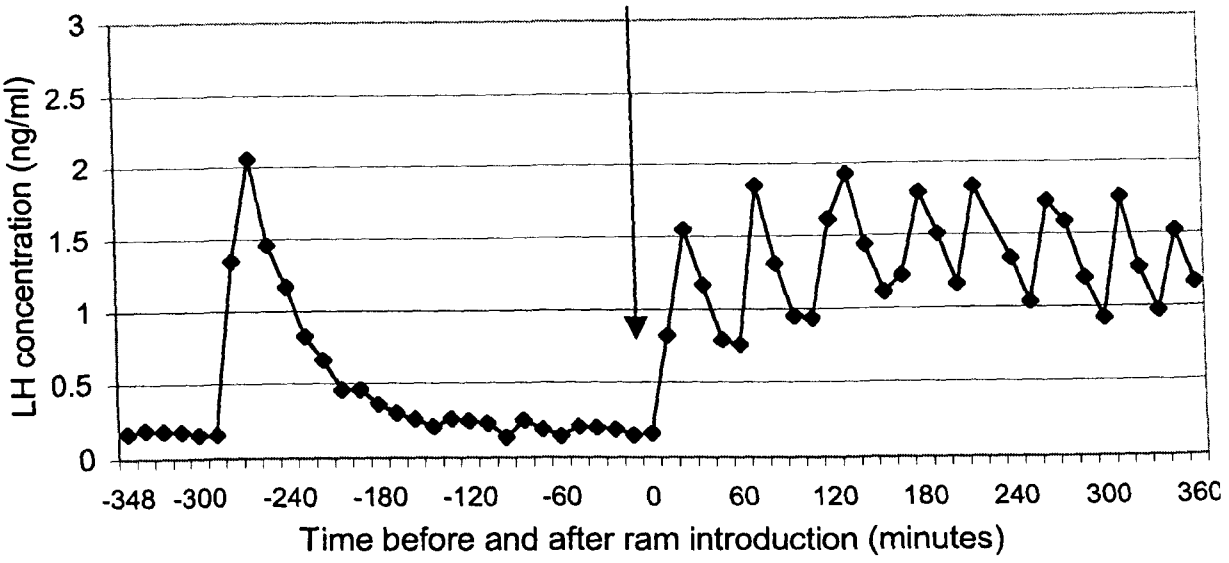
Table 6.1 Luteinising hormone characteristics of ram naive (RN) and ram experienced (RE) cyclic maiden ewes before and after ram introduction during the breeding season. One RE ewe was excluded from analysis as was on the down slope of an LH surge at the onset of the frequent blood-sampling period. Values are presented as mean ± S.E.M. Values differ from before ram introduction within the treatment group; $ P<0.1; *P<0.05; **P<0.01; ***P<0.001.

		RN	RE
Number of ewes		6	5
LH parameters before and after ram introduction			
Mean LH concentration (ng/ml)	Before rams	0.31± 0.05	0.30 ± 0.06
	After rams	0.77± 0.09**	1.00 ± 0.08**
Mean number of LH pulses in 6 hours	Before rams	1.60± 0.40	1.33 ± 0.67
	After rams	3.80± 0.67$	3.33 ± 0.37*
Mean LH pulse amplitude (ng/ml)	Before rams	0.74± 0.34	0.41± 0.16
	After rams	0.48± 15	0.83± 0.27
Mean basal concentration of LH (ng/ml)	Before rams	0.14± 0.01	0.17± 0.05
	After rams	0.47± 0.12*	0.77± 0.25*

Table 6.2. Changes in LH concentrations and the number and amplitude of pulses of LH during the 6-hour period before and after ram introduction. Values for ewes in their follicular and mid luteal phase are included however no statistical analysis could be carried out due to only one ewe being present per group. Data are presented as mean ± sem. Values differ from before ram introduction within stage of cycle $ P<0.1, *P<0.05, **P<0.01, ***P<0.001.

		Follicular	Early luteal	Mid luteal	Late luteal
	Number of ewes	1	3	1	6
LH parameters before and after ram introduction					
Mean LH concentration (ng/ml)	Before rams	0.53	0.28 ± 0.10	0.16	0.31 ± 0.06
	After rams	0.75	1.13 ± 0.42$	0.70	0.83 ± 0.21**
Mean LH pulses per 6 hours	Before rams	4.00	1.67 ± 0.67	No pulses	1.17 ± 0.43
Equivalent per hour		(0.67)	(0.28)	(0)	(0.20)
	After rams	4.00	3.33 ± 0.67$	3.00	3.67 ± 0.79*
		(0.67)	(0.60)	(0.50)	(0.62)
Mean LH pulse amplitude (ng/ml)	Before rams	0.64	0.53 ± 0.05	No pulses	0.69 ± 0.43
	After rams	0.41	0.46 ± 0.20	1.94	0.61 ± 0.18
Mean basal LH concentration (ng/ml)	Before rams	0.33	0.17 ± 0.02	0.15	0.10 ± 0.03
	After rams	0.64	0.95 ± 0.37*	0.19	0.56 ± 0.16*

LH profile



Progesterone profile

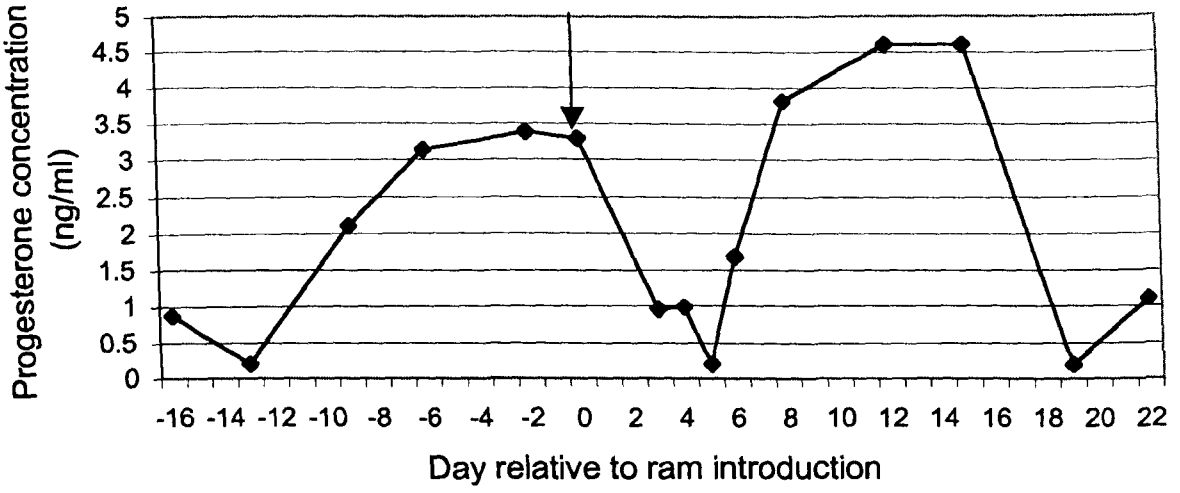
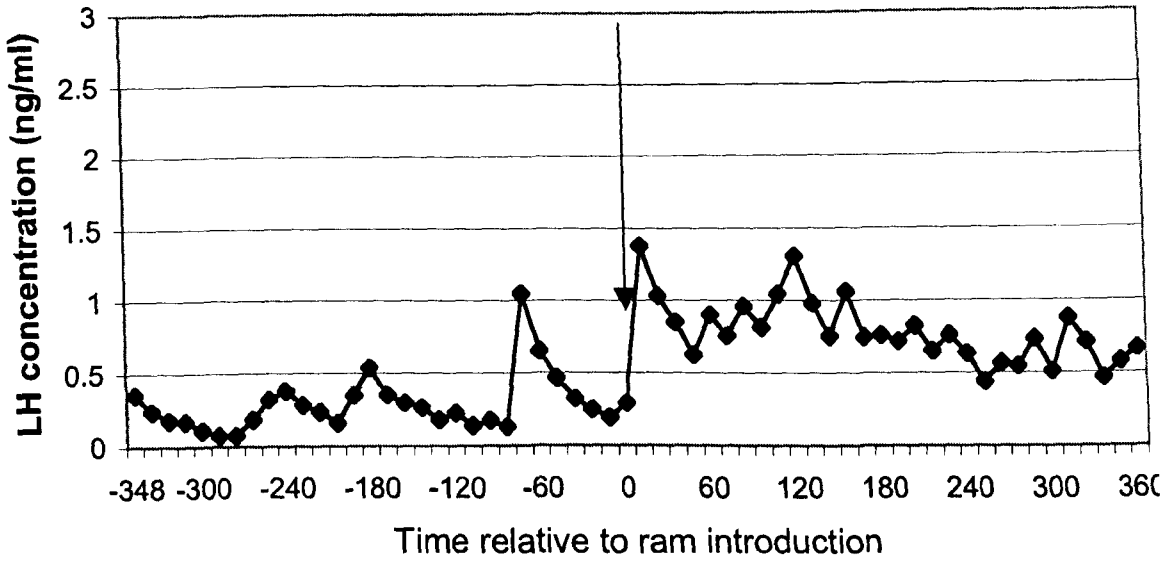


Figure 6.2 Representative profile of a ewe (ewe 23) in the late luteal phase on the day of ram introduction. Ewe 23 responded to ram introduction with an increase in LH concentrations driven by a marked increase in LH pulse frequency. An LH surge was detected 37 hours after ram introduction but the duration could not be recorded, as the circulating concentrations of LH did not return to basal within the blood-sampling period (19-43 hours after ram introduction).

LH profile



Progesterone profile

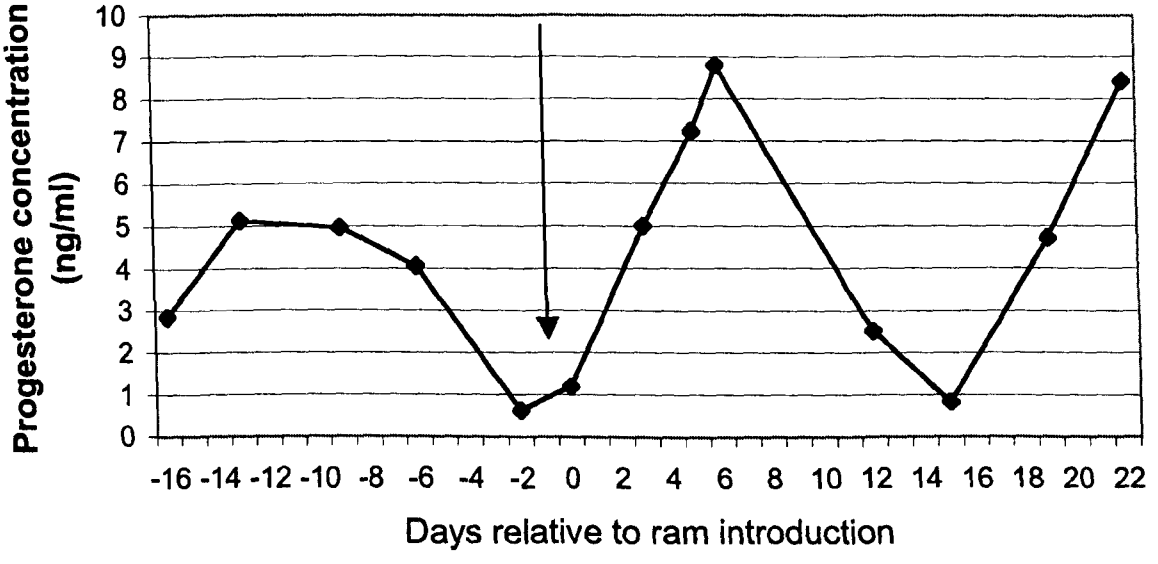
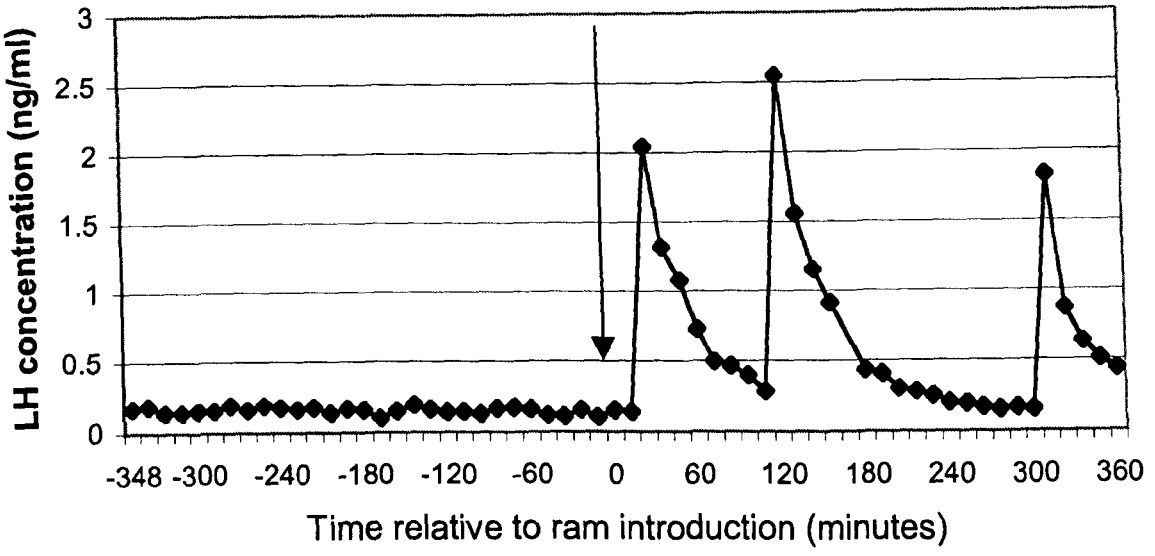


Figure 6.3 Representative profile of a ewe (ewe 19) in the early luteal phase on the day of ram introduction. Ewe 19 responded to ram introduction with an increase in LH concentrations however no LH surge was detected in the blood-sampling period between 19 and 43 hours after ram introduction.

LH profile



Progesterone profile

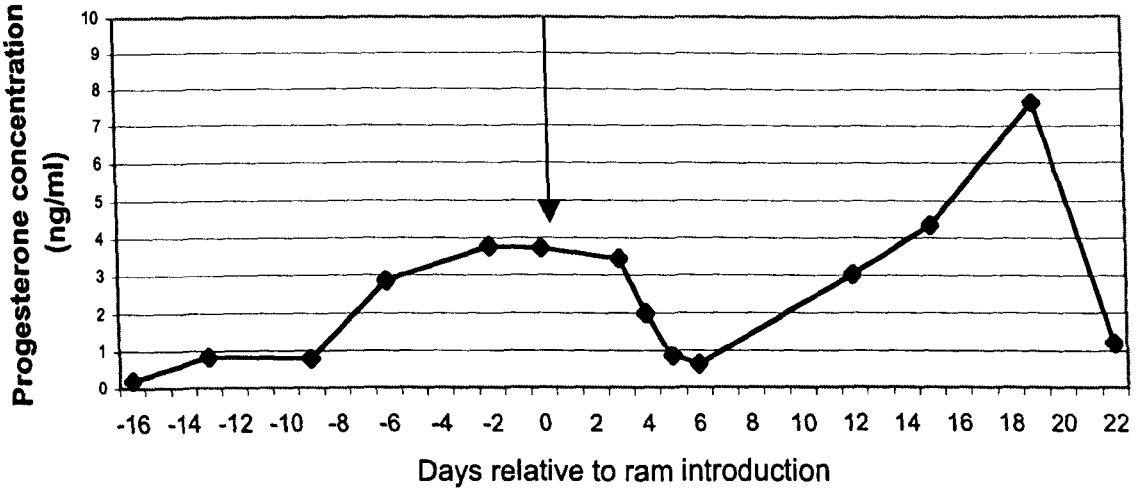
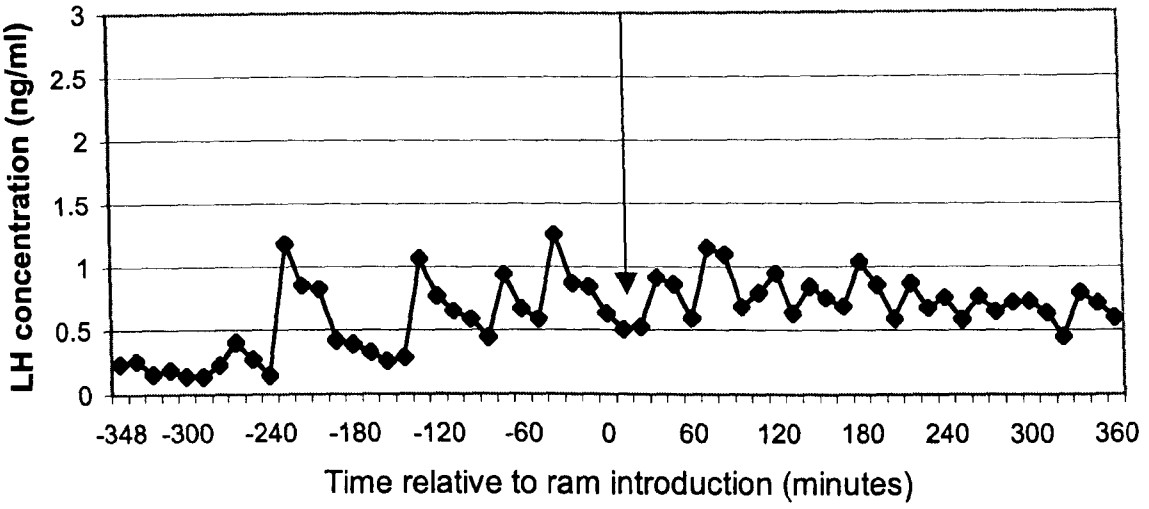


Figure 6.4 Representative profile of a ewe (ewe 16) in the mid luteal phase on the day of ram introduction. Irrespective of the high circulating concentrations of progesterone it is apparent that ewe 16 still responded to ram introduction as shown above. As expected in a ewe at this stage of oestrous cycle, no LH surge was detected in the blood-sampling period

LH profile



Progesterone profile

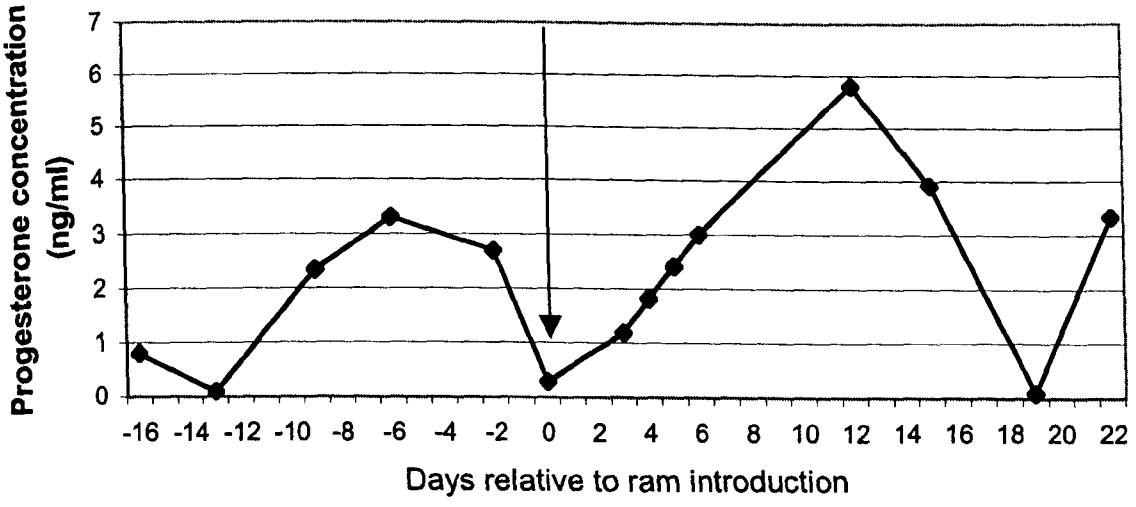
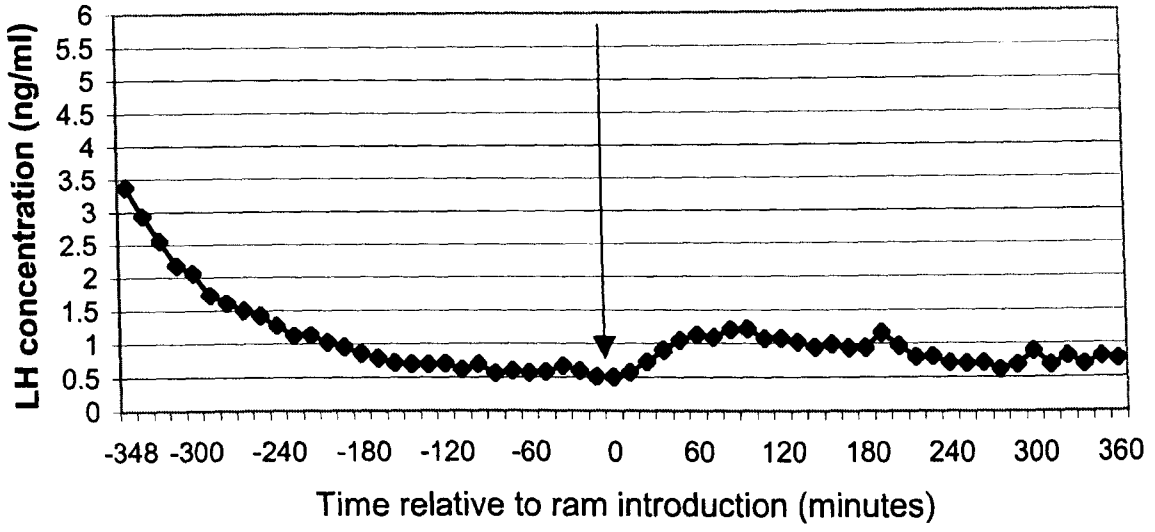


Figure 6.5 Representative profile of a ewe (ewe 18) in the follicular phase on the day of ram introduction. Ewe 18 responded to ram introduction with an apparent increase in LH pulse frequency. However the 12-minute sampling period was insufficient to detect definitive pulses due to the high pulse frequency observed in the follicular phase. The onset of the LH surge occurred prior to beginning of the sampling period and ended at 33 hours after ram introduction.

LH profile



Progesterone profile

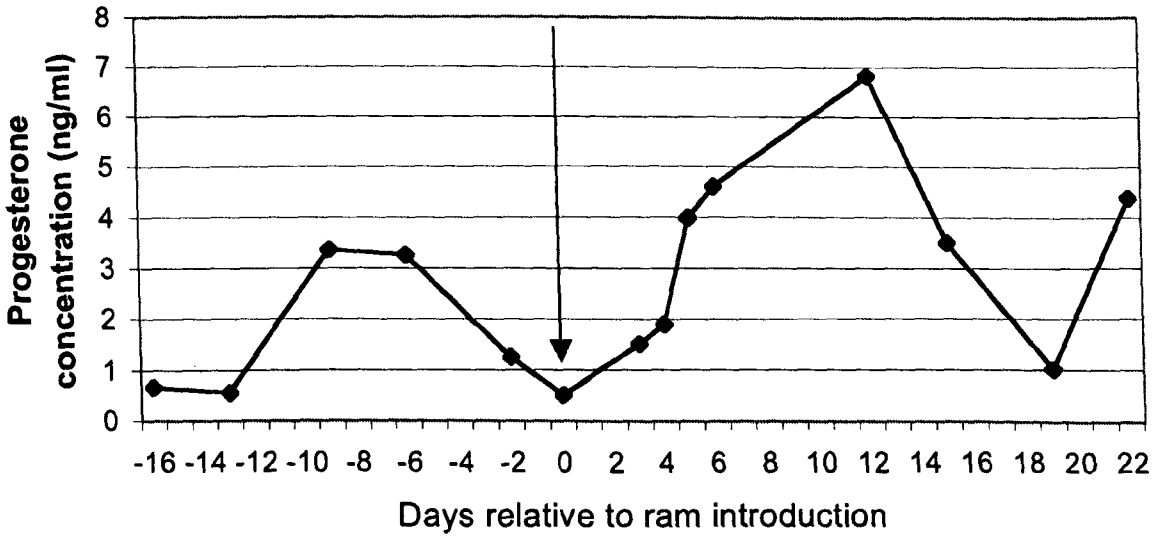


Figure 6.6 Representative profile of a ewe (ewe 21) that appears to be on the down slope of an LH surge on the day of ram introduction and was therefore excluded from the LH analysis. However this ewe is included here to note the marginal increase in basal concentrations of LH after ram introduction as denoted by the arrow.

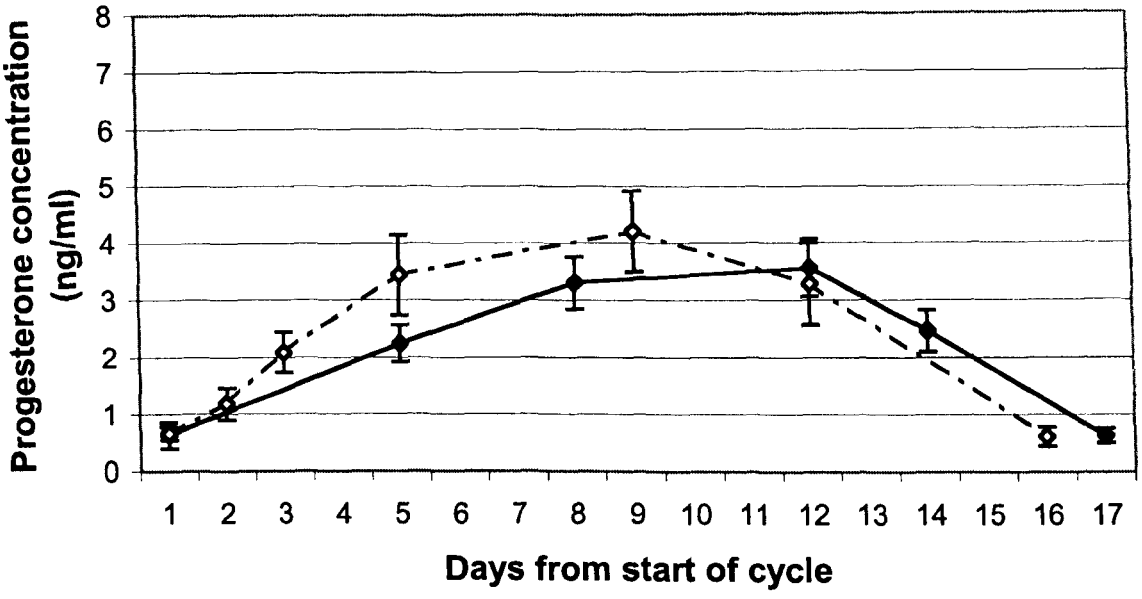


Figure 6.7 Cycle length, shape and distribution of the oestrous cycles of maiden ewes in their late luteal phase on the day of ram introduction (n=6). During the first oestrous cycle (solid line, closed diamond) ewes had a mean cycle length of 17.17 ± 0.65 days and the date of ram introduction fell on Day 14 (n=5 ewes) or Day 17 (n=1 ewe). The rams remained with the ewes for the duration of the second and into a third cycle. During the second oestrous cycle (dashed line, open diamond) ewes had a mean cycle length of 15.83 ± 0.54 days, however this reduction in cycle length only tended towards significance ($P \leq 0.1$).

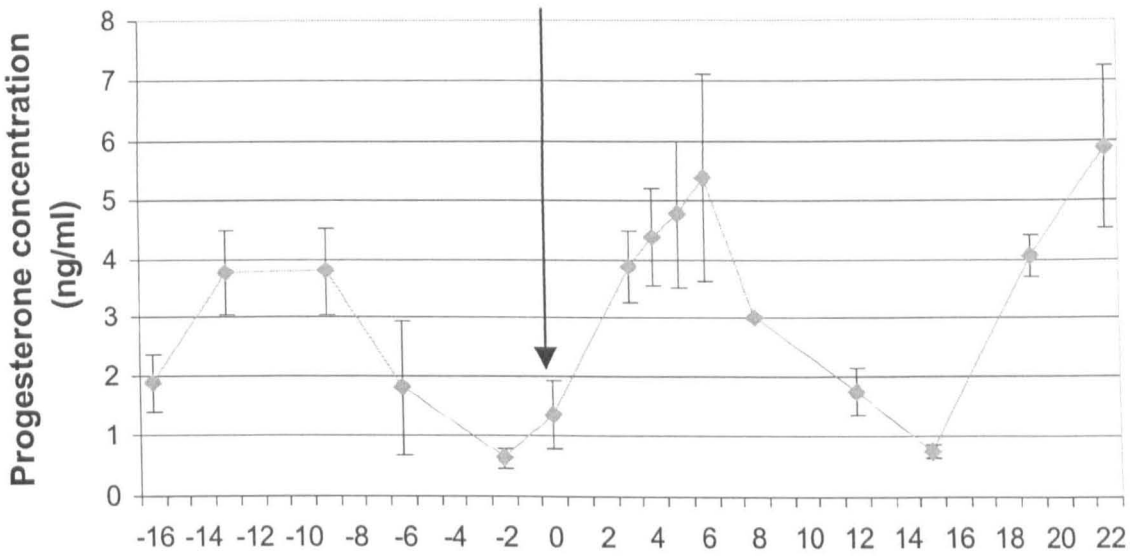


Figure 6.8. Mean distribution (± sem) of oestrous cycles of ewes in their early luteal phase on the day of ram introduction indicated by the arrow above (n=3). Cycle length of the cycle during ram introduction was 17.67 ± 0.67 days. Cycle length of the cycle before or after this cycle could not be calculated due to the absence of more than two successive a nadir reference points.

6.5 DISCUSSION

Cyclic maiden ewes introduced to rams during the breeding season respond to ram introduction with an increase in LH concentrations that is independent of the stage of the oestrous cycle at the time of ram introduction. Prior experience with the ram does not appear to modulate any aspects of the endocrine response of cyclic maiden ewes to ram introduction during the breeding season.

The absence of any effect of prior experience with the ram on the endocrine response of maiden ewes to ram introduction is in agreement with that observed during anoestrus in Chapter 5. I propose that the depression in fertility characteristic of maiden ewes first mated during the breeding season (Dyrmundsson, 1973) is most likely to be due to behavioural aspects of ram-ewe interactions as proposed by Rosciszewska, (1985) and Gelez *et al.,* (2004a) rather than a deficiency in the endocrine response to the ram.

The capacity of the ram to induce an increase in LH concentrations in ewes in both the early and late luteal phase is similar to that observed in ewes introduced to rams whilst under the influence of artificial progestagen (Martin *et al.,* 1983; Pearce and Oldham, 1983; Evans *et al.,* 2004). The ram induced increase in LH pulse frequency, circulating and basal concentrations of LH within ewes in their late luteal phase concurs with observations of Al-Gubory (1998). Ewes introduced to rams on Day 10 after mating had a significant increase in LH pulse frequency (before rams; 1.4 ± 0.2 after rams; 2.8 ± 0.4 pulses per 4 hours) that is comparable to that observed in this study in non-pregnant ewes in their late luteal phase at the time of ram introduction (before rams; 0.8 ± 0.3 to after rams; 2.5 ± 0.5 pulses per 4 hours).

During the luteal phase of the oestrous cycle, LH release is constrained by a negative effect of progesterone and oestradiol on GnRH release from the hypothalamus (Karsch *et al.,* 1977). The capacity of the ram to induce an increase in parameters of LH release during the luteal phase indicates an override or circumvention of the steroidgenic control of LH release. Previous studies have shown a critical role of opioids in mediating the steroidgenic inhibition of LH release (Brooks *et al.,* 1986) through a direct effect on the GnRH pulse generator (van Vugt, 1985). Relative to the increase in

LH pulse frequency observed in this study, Stansfield *et al.*, (1987) identified a role of endogenous opioids in the successful stimulation of the ram effect in prepubertal ewe lambs. They proposed that the chemosignals from the ram induced an early depletion of the inhibitory control of the GnRH pulse generator permitting an LH response to ram introduction (Stansfield *et al.*, 1987). To our knowledge no further studies have investigated the role of opioids in mediation of the ram effect. However the observation that main olfactory bulb neurons connect with the preoptic and medial basal regions of the hypothalamus (Jansen *et al.*, 1998) indicates the potential for a direct effect of the socio-sexual cues of the ram on GnRH release. Whisnant *et al.*, (1991) investigated the effects of antagonism of opioids during the luteal phase to determine the role of opioids in controlling the frequency and amplitude of GnRH release. Intrahypothalamic implantation of an endogenous opioid antagonist (WIN 44,441-3) into the preoptic area and medial basal hypothalamus during the luteal phase caused an increase in GnRH pulse frequency, thus indicating a direct role of opioids in mediating the steroidgenic suppression of LH during this part of the oestrous cycle. Therefore I propose that the increase in LH pulse frequency after ram introduction in ewes in both their early and late luteal phase may be mediated through a direct action of socio-sexual cues from the ram on the opioid control of GnRH release.

The observation in this study of a negligible effect of ram introduction on pulse amplitude whilst under the influence of endogenous progesterone is in agreement with that of Al-Gubory (1998) where there was no effect of ram introduction on LH pulse amplitude when rams were introduced to ewes 10 days post mating. However it is of interest to note that pulse amplitude in this study both before (0.69 ± 0.43ng/ml) and after ram introduction (0.61 ± 0.18 ng/ml) was markedly lower than that observed by Al-Gubory (1998; before rams; 1.63 ± 0.56, after rams; 1.55 ± 0.26). This may be due to differences in the stage of the luteal phase during ram introduction, plane of nutrition prior to ram introduction or a characteristic of pregnant ewes.

Ewes in their late luteal phase showed a significant decline in progesterone concentrations between the beginning and end of the serial bleed period. This observation may be a reflection of the natural decline in LH during the late luteal phase. Luteolysis in naturally cycling ewes typically spans 2 to 3 days (Wiley *et al.*,

1997) with an average daily decline in progesterone of approximately 1 ng/ml (Acritopoulou *et al.*, 1977). The mean decline in progesterone concentrations over the 12-hour sampling period in this study was 0.69 ± 0.21 ng/ml that is marginally above this estimate when considered over a 24 hour period. This observation in addition to the variation within ewes classified in their late luteal phase (minimum decline: 0.25ng/ml; maximum decline: 1.64ng/ml over the 12 hour period) suggests the possibility of an enhanced rate of decline related to ram introduction. Recent *in vitro* work has shown an up-regulation in LH receptors in the porcine, bovine and ovine endometrium before and at the time of luteolysis (Review; Ziecek *et al.*, 2004) followed by a down regulation after Day 16 of the oestrous cycle (Stepien *et al.*, 1999). The authors proposed a luteolytic role of LH at the end of the oestrous cycle based on increased secretion of $PGF_{2\alpha}$ in endometrial cells exposed *in vitro* to LH (Ziecek et al., 2004) and stimulated expression of the enzyme critical to prostaglandin production (COX-2; Stepien, 1999). Zarco *et al.*, (1988) identified the time of the onset of an increase in $PGF_{2\alpha}$ pulse frequency to be the main determinants of cycle length in the ewe. Crucially in the porcine endometrium, Ziecek *et al.*, (2001) identified a high level of agreement between LH and $PGF_{2\alpha}$ peaks (79.2%) thus suggesting that an increase in LH pulse frequency has the potential to directly affect the occurrence and frequency of endogenous $PGF_{2\alpha}$ release (Ziecek *et al.*, 2004). Therefore it is possible that an increase in mean and specifically pulsatile LH secretion induced by ram introduction during the late luteal phase may affect the rate of progesterone decline and time of luteolysis of the corpus luteum through a direct effect on $PGF_{2\alpha}$ release.

The absence of a significant effect of stage of the luteal phase at the time of ram introduction indicates that a similar endocrine response to ram introduction occurs irrespective of whether ewes are in their early or late luteal phase. However it is the subsequent physiological effect of the ram-induced increase in LH concentrations that may differ between these two phases of the oestrous cycle. Elevated concentration of LH during the early luteal phase is critical for the development of a competent CL and progesterone production from the small LH dependent luteal cells (Niswender *et al.*, 1994). Therefore as inadequate gonadotrophic support can be a causal factor in the development of short-lived corpora lutea (Review; Garverick *et al.*, 1992), enhanced endogenous LH at this stage of oestrous cycle is likely to affect luteal development and

thus progesterone production. Therefore exposure of the developing corpus luteum to supplementary LH released in response to ram introduction may enhance the potential and actual progesterone production during the luteal phase of the oestrous cycle. Progesterone concentrations during the early luteal phase are a critical determinant of oestrous cycle length in the ewe (Nephew *et al.*, 1991). Ewes with shorter length oestrous cycles (15.9 ± 0.1 versus 18.6 ± 0.4 days) have been found to have higher levels of progesterone secretion on Days 2, 3 and 4 of the oestrous cycles than ewes exhibiting long oestrous cycles. This suggests a causal relationship between progesterone release during this early phase and oestrous cycle length (Nephew *et al.*, 1991).

In relation to ewes in their late luteal phase at the time of ram introduction, the physiological effects of elevated LH during declining concentrations of progesterone may to be similar to those seen in artificially synchronised ewes (Flynn *et al.*, 2000; Evans *et al.*, 2004; Hawken *et al.*, 2005). Ram introduction towards the end of a progestagen protocol stimulates an increase in LH concentrations (Evans *et al.*, 2004). The capacity of these increased LH concentrations to affect both follicle development and parameters of LH release after progestagen withdrawal have been shown by Evans *et al.*, (2004) and Hawken *et al.*, (2005). These results indicate the potential for the LH increase observed in this study to affect aspects of follicle development, ovulation rate and the timing of the LH surge and ovulation. Furthermore the ewe in the mid-luteal phase and thus under the influence of optimal progesterone concentrations (Figure 6.4) showed a numerical increase in LH concentrations, pulse frequency, pulse amplitude and basal concentration of LH thus emphasizing the potency of the ram stimulus in eliciting this type of response. The physiological relevance of increased LH at this stage of the oestrous cycle would require further investigation.

In summary, maiden ewes with or without prior experience with the ram respond to ram introduction during the breeding season with an endocrine response that is independent of the stage of the oestrous cycle at the time of ram introduction. There is no significant effect of stage of the luteal phase on any parameters of the LH response and ewes under the influence of endogenous progesterone respond to ram introduction with an increase in LH pulse frequency, basal and mean concentrations of LH. The capacity of ewes, irrespective of stage of the oestrous cycle to respond to ram

introduction with an LH response reiterates the potency of the socio-sexual cues of ram in mediating physiological changes in the reproductive activity of the ewe.

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7. INVESTIGATION INTO THE EFFECT OF RAM INTRODUCTION DURING THE LATE ANOESTRUS ON THE ENDOCRINE RESPONSES OF MAIDEN EWES WITH OR WITHOUT PRIOR EXPERIENCE OF THE RAM DURING THE PREVIOUS BREEDING SEASON

7.1 ABSTRACT

The pre-exposure period for ram experienced ewes in Chapters 5 and 6 was timed during anoestrus and was of insufficient duration for any ewes to be in oestrus during the period of ram presence. Based on the importance of mounting and intromission in the development of experience and proficiency of behavioural interactions, I propose that pre-exposure of maiden ewes to rams during the breeding season will be a more potent stimulus and modify the endocrine response of maiden ewes to rams. At the end of November maiden ewes were either kept with vasectomised rams for 1 month (MRB; ram experienced; n=10) or isolated from ram contact (MCB; ram naïve; n=10). All ewes were subsequently maintained in isolation from ram contact. During late anoestrus (the following August) MRB (n=10) and MCB (n=10) ewes were introduced to rams midway through a frequent blood sampling regime. Both MRB and MCB ewes had a significant increase in mean and basal concentrations of LH and LH pulse frequency ($P<0.001$) however there was no significant effect on LH pulse amplitude. MCB ewes had significantly greater LH pulse frequency than MRB ewes both before ($P<0.05$) and after ($P<0.01$) ram introduction. There was no effect of prior experience of the ram on any other parameters of the LH response ($P>0.1$). Numerically more MCB ewes were detected with an LH surge between 18 and 42 hours after ram introduction ($P<0.1$) and MCB ewes had a significantly earlier onset of the LH surge than MRB ewes. I propose that the endocrine response to the ram is not enhanced by prior exposure to the ram during the breeding season.

7.2 INTRODUCTION

Rams detect ewes in heat by actively pursuing the ewes and investigating the anogenital region of the ewes (Kelley, 1937). The intensity of ram to ewe contact (driven by ram libido and the level of expression of sexual behavior) is known to affect the elicitation of the LH response in anoestrous ewes induced to ovulate with the ram effect (Perkins and Fitzgerald, 1994). Within Chapters 5 and 6, maiden ewes were classified as ram experienced after a seven-day preconditioning ram exposure period during mid anoestrus. However based on the length of this exposure period and the well established 18-25 day delay to behavioural oestrus in anoestrous ewes stimulated by the ram effect (Pearce and Oldham, 1984), no ewes would have been in oestrus during this preconditioning exposure period. Therefore this would have prevented the close investigation and mounting associated with ewe-ram contact when ewes are in oestrus.

Exposure of rams to oestrous ewes causes an increase in mean LH and testosterone concentrations in rams (Gonzalez *et al.*, 1988) and improves the vigour and expression of sexual behavior of the ram (Rosa *et al.*, 2000). Therefore the exposure of sexually naïve maiden ewes to vasectomised rams during the breeding season may be more likely to result in a greater potency of the ram stimulus relative to increased pheromone transmission, tactile contact and observations of sexual behaviour. Furthermore Gelez *et al.*, (2004a) hypothesised that the levels of proceptivity and receptivity associated with the oestrous behaviour of an adult sexually experienced ewe develops over a number of mounting and intromission experiences. This theory is based on the absence of a specific pattern of dopamine and noradrenalin release in the medial basal hypothalamus around the time of oestrus in sexually naïve ewes (Gelez *et al.*, 2004a). If the preconditioning exposure period were to occur during the breeding season, the maiden ewes are likely to be mounted by the rams thereby developing this neuroendocrine response and improving the behavioral responses of the maiden ewes.

The aim of this experiment was to investigate the effect of pre-exposure of maiden ewes to a vasectomised ram for a month during the breeding season on their subsequent endocrine responses to ram introduction during late anoestrus. I aimed to detect any differences in the LH response of ram experienced and ram naïve ewes and

any differences in the proportion of ewes having an LH surge and ovulating in response to ram introduction. I also aimed to detect any differences in the latency to cyclic activity, oestrous cycle length and maximum progesterone concentration.

7.3 MATERIALS AND METHODS

7.3.1 PRE-EXPERIMENTAL PROTOCOL

Sexually naïve spring born maiden mule ewes (Swaledale x Bluefaced/Border Leicester) that had been previously isolated from ram contact (not within 500m) were randomly assigned to either control (MCB; n=10) or ram experienced groups (MRB; n=10) and maintained on pasture at Cockle Park Research Farm (55°13'N). At the end of November, MRB ewes (age; approximately 7 months) were introduced to vasectomised rams (n=6) for a period of 1 month. MRB and MCB ewes were subsequently isolated from ram contact and maintained together at pasture in accordance with conventional farm practice until August the following year (Figure 7.1). As in Chapters 5 and 6, all rams used were vasectomised rams but will be referred to as rams throughout.

7.3.2 ANIMALS AND EXPERIMENTAL PROCEDURES

During August, MRB (n=10) and MCB (n=10) ewes were split into two replicates (1; n=10, 2; n=10) containing an equal number of randomly selected ewes from each treatment group (Figure 7.1). The second replicate was staggered by 2 days to permit the same frequency of blood sampling as in Chapters 5 and 6 (every 12 minutes) with the limited amount of technical support available. The minimal two-day gap limited the likelihood of any seasonal effects on the ewe-ram response. MRB1 (n=5) and MCB1 (n=5) ewes were mixed and housed in 2 groups of 5 ewes per pen (19m^2 per pen) and kept under an artificial controlled lighting regimen, which matched the natural day length. In a similar controlled environment, MRB2 (n=5) and MCB2 (n=5) ewes were mixed and housed in 2 groups of 5 ewes per pen (14m^2 per pen) and maintained under natural lighting. Each group received hay (3kg) and a daily ration of concentrates (300g per ewe).

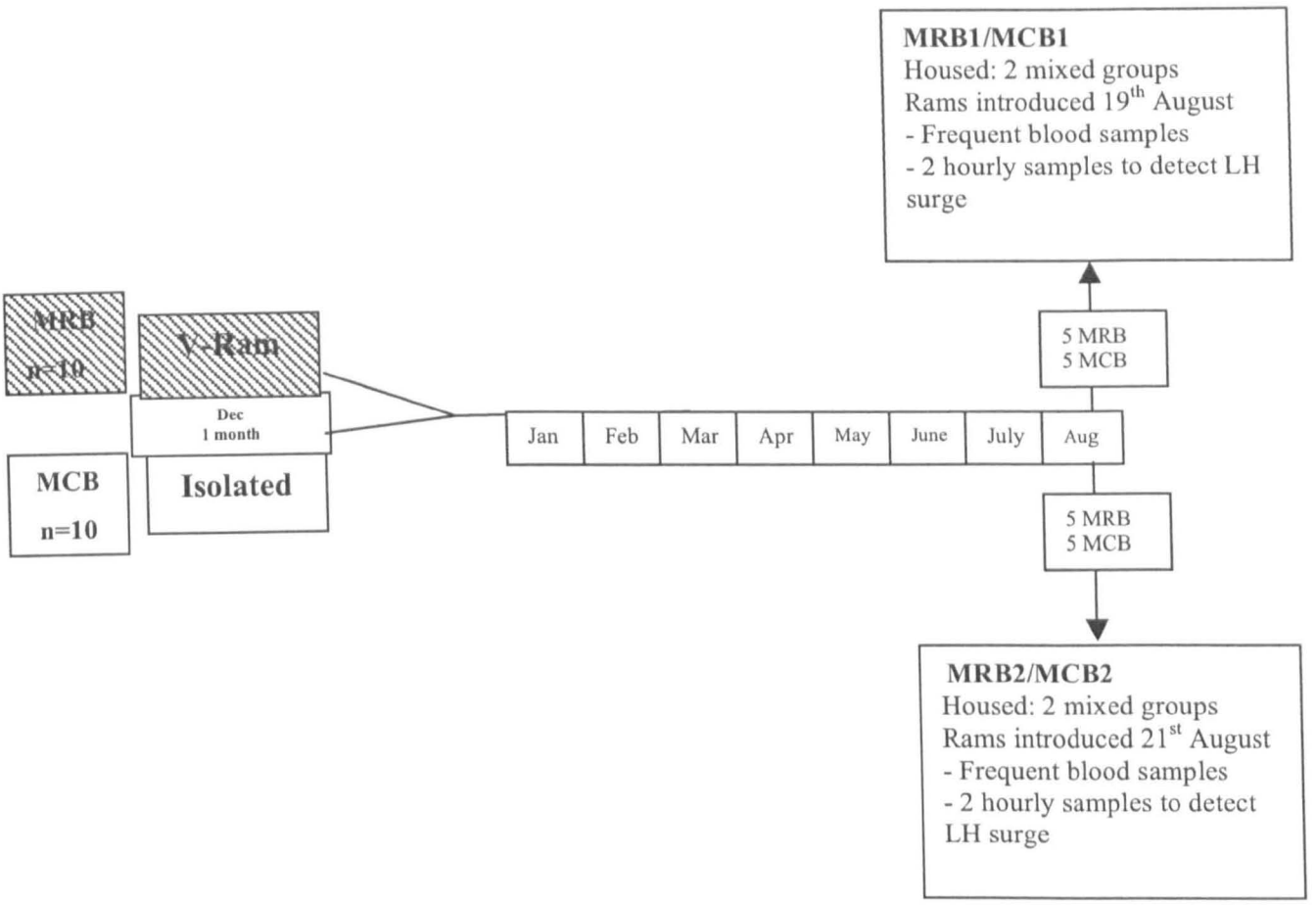


Figure 7.1. On Day 0 of the experiment (MRB1/MCB1, 19th August; MRB2/MCB2, 21st August), rams were introduced midway through the serial bleed procedure outlined below and remained with the ewes for the remainder of the experiment.

7.3.3 CANNULATION PROCEDURE AND BLOOD PROCESSING

The cannulation procedure, maintenance of the cannulae and processing of the blood samples were carried out as in Chapter 5.3.4.2 and 5.3.5

7.3.4 BLOOD COLLECTION

On the day of ram introduction (Day 0), frequent blood samples (5ml) were collected every 12 minutes via a jugular cannula during the 6 hours before and 6 hours after ram introduction. On Day 1 after ram introduction, blood samples were taken every 2 hours via the jugular cannula from 18 to 42 hours after ram introduction to attempt to detect the LH surge.

Blood samples (5ml) were collected by jugular venepuncture (Vacutainer, Becton-Dickinson Limited, Coventry) or via the cannula for progesterone in order to establish that the ewes were anoestrus prior to ram introduction and to monitor their progesterone profiles. As in Chapters 5 and 6, blood samples were taken twice weekly for 2 weeks prior to ram introduction with the frequency increased to daily during Days 3 to 6 after ram introduction. On Day 6 the cannulae were removed and the frequency of samples rescheduled to twice weekly via jugular venepuncture until three weeks after ram introduction.

7.3.5 IMMUNOASSAY

Plasma progesterone concentrations were analysed in duplicate using a commercial enzyme linked immunoassay (ELISA) kit (Ridgeway Science Ltd, Gloucester, UK) as outlined in Chapter 5.3.5. Mean intra-assay and inter-assay coefficients of variation for low (1.77ng/ml), medium (2.98ng/ml) and high (7.23ng/ml) plasma samples were 8.3% and 12.2% and 5.4 and 10.2% and 6.3 and 14.1% respectively. The sensitivity of the assay was 0.2ng/ml.

Serum LH concentrations were determined using a previously validated double antibody radioimmunoassay as outlined in Chapter 5.3.6. Mean intra-assay and inter-assay coefficients of variation for low (0.22ng/ml), medium (1.50ng/ml) and high (3.39ng/ml) plasma samples were 6.1% and 15.3%, 7.4% and 10.2% and 7.4% and 15.7% respectively. The sensitivity of the assay was 0.1ng/ml.

7.3.6 DATA ANALYSIS

Any ewe classed as cyclic prior to ram introduction (determined by progesterone elevation above 1.5ng/ml for at least 2 samples) was excluded from further analysis. The onset of cyclic activity post ram introduction was determined as the sample when the progesterone concentration was elevated by a minimum of two standard deviations above the mean of the previous samples and remained elevated for two or more consecutive samples. Cycle length was derived as the number of days between two successive nadir points on the progesterone profile.

Serum LH concentrations were plotted out for individual ewes over the duration of the 12 hour blood sampling period. As the secretion of LH was pulsatile, the

frequency of pulses was analysed using the Munro algorithm, which is a modified version of the Pulsar algorithm (Wachter and Merriam, 1982). The parameters for the Munro analysis were the same as those outlined in Chapter 5.3.7. Data for LH pulse frequency, LH pulse amplitude, mean and basal LH concentrations before and after ram introduction were subject to repeated measures ANOVA in Genstat 5 (for Windows, Second Edition) as in Chapters 5.3.7. Mean and basal LH were log10 transformed due to skewed distribution of the data however the non-transformed data are presented for ease of interpretation. Where a significant effect of prior experience of the ram was detected, data for MRB and MCB ewes were compared before and after ram introduction by Students t-test (Minitab, 13.1).

Occurrence of an LH surge was determined as outlined in Chapter 5.3.7. The number of ewes in each treatment group having an LH surge was compared by a Chi Square test and the time of the onset, end and duration of the LH surge were compared between treatments by Students t-test. The number of LH pulses during the 6-hour period both before and after ram introduction were compared between ewes detected with or without an LH surge using Mann Whitney *U* test due to the abnormality of the data.

The progesterone concentrations on Day 3 after ram introduction (the first progesterone sample taken after ram introduction) and the number of days to the onset of cyclic activity were compared between treatments by Students t-test after log 10 transformation. As above the non-transformed data are presented for ease of interpretation.

Cyclic activity after ram introduction was categorized using each ewe's progesterone profile into one of four categories (outlined in Chapter 5.3.7). The numbers of ewes exhibiting each classification of post-ram cyclic activity were compared using the Chi Square test.

7.4 RESULTS

Based on the progesterone data, all ewes were anoestrus at the onset of the serial bleed with the exception of one MCB and two MRB ewes, which for this reason were excluded from further analysis. Replicate was included in the repeated measures analysis and was found not to have a significant effect on (or interaction with) any parameters associated with the LH response and thus MCB and MRB ewes from each replicate were combined for further analysis.

Ram introduction during late anoestrus to maiden ewes with (ram experienced; MRB) or without (ram naïve; MCB) prior experience of the ram stimulus during the previous breeding season induced a significant increase in mean (Table 7.1; $P<0.001$) and basal concentrations of LH (Table 7.1; $P<0.001$) and LH pulse frequency (Table 7.1; $P<0.001$). There was no significant effect of ram introduction on LH pulse amplitude (Table 7.1; $P>0.1$).

MCB ewes had significantly greater LH pulse frequency than MRB ewes during the 6-hour sampling period before ram introduction (Table 7.1; $P<0.05$). After ram introduction, MCB ewes continued to have a greater number of LH pulses during the 6-hour sampling period (Table 7.1; $P<0.01$) than MRB ewes. There was no interaction between prior experience of the ram and the LH response to ram introduction (Table 7.1; $P>0.1$). There was no significant difference between MCB and MRB ewes in any other parameters of the LH response (mean and basal LH concentrations or LH pulse amplitude) either before or after ram introduction (Table 7.1; $P>0.1$).

An LH surge was detected within the blood-sampling period between 18 and 42 hours post ram introduction in numerically more MCB ewes than MRB ewes (8 versus 4; $P<0.1$). Table 7.1 shows the differences between the MCB and MRB ewes in the latency to the onset of the LH surge. However the proportion of MCB ewes that had the onset of the LH surge prior to 18 hours after ram introduction prevented any statistical analysis on this data (n=3; 38% of MCB ewes detected with a surge). Similarly the number of MRB ewes that had the end of the LH surge outside of the sampling period (n=2; 50% of MRB ewes detect with a surge) prevented meaningful analysis between MCB and MRB on the end or duration of the LH surge.

Within MRB and MCB ewes there were no differences in mean LH concentrations before and after ram introduction in ewes detected with or without an LH surge between 18 and 42 hours after ram introduction (Figure 7.2 and 7.3). MRB ewes subsequently detected with an LH surge had significantly more LH pulses after ram introduction than MRB ewes not detected with an LH surge during the sampling period (Figure 7.4; $P<0.05$). This difference could not be assessed statistically in MCB ewes due to only one ewe not detected with an LH surge (Figure 7.5). Representative individual profiles for MRB and MCB ewes that were detected with or without an LH surge between 18 and 42 hour after ram introduction are shown in Figures 7.7-7.8

In the absence of a significant difference between ram experienced and ram naïve ewes either before or after ram introduction, data for MRB and MCB was pooled and re-categorized as ewes detected with or without an LH surge. Ewes detected with an LH surge had significantly greater mean LH concentrations before ram introduction than ewes not detected with an LH surge within the sampling period (Figure 7.8; $P<0.05$). However there was no significant difference in mean LH concentrations after ram introduction.

The mean onset of cyclic activity occurred significantly earlier in MCB ewes (Table 7.1; $P<0.01$). This is likely to be related to the earlier onset of the LH surge in the MCB ewes (Table 7.1). There were no significant differences in the remaining characteristics of the oestrous cycles post ram introduction (Table 7.1).

Table 7.1. Luteinising hormone characteristics of ram naive (MCB) and ram experienced (MRB) anoestrous maiden ewes before and after the introduction of vasectomised rams. Values are presented as mean ± S.E.M. Value differs from before ram introduction within each treatment (***P<0.001). Within rows different superscripts indicate a significant difference between ram naïve and ram experienced ewes (ef; P<0.05, gh P<0.01)

		MCB	MRB
Number of ewes		9	8
LH parameters before and after ram introduction			
Mean LH concentrations (ng/ml)	Before rams	0.43 ± 0.1	0.28 ± 0.06
	After rams	2.21 ± 0.33***	1.75 ± 0.20***
Mean number of LH pulses in 6 hours	Before rams	1.22 ± 0.15e	0.63 ± 0.18f
	After rams	5.89±0.35g***	4.13 ± 0.55h***
Mean LH pulse amplitude (ng/ml)	Before rams	1.67 ± 0.32	1.55 ± 0.32
	After rams	1.87 ± 0.35	2.44 ± 0.36
Mean basal level of LH (ng/ml)	Before rams	0.19 ± 0.06	0.15 ± 0.05
	After rams	1.56 ± 0.37***	0.90 ± 0.15***
LH surge parameters			
Ewes having LH surge (%)		8 (89)	4 (44)
Time to LH surge (Hrs after ram introduction) (Number of ewes with onset after 18 hrs)		23.2 ± 1.62 (5)	29.0 ± 2.38 (4)
End of LH surge (Hrs after ram introduction) (Number of ewes with end within 42hrs)		37.5 ± 1.95 (8)	41.0 ± 1.0 (2)
Duration (Hrs) (Number of ewes with onset and end within sampling period)		17.6 ± 0.75 (5)	16.0 ± 2.0 (2)
Oestrous cycle parameters: *After ram introduction, ewes having:*			
Short cycle only		0	0
Short cycle then long cycle		7	5
Long cycle only		2	1
Not cycling by 21 days post ram introduction		0	2
Mean number of days to cyclic activity		3.67 ± 0.24g	6.14 ± 1.16h

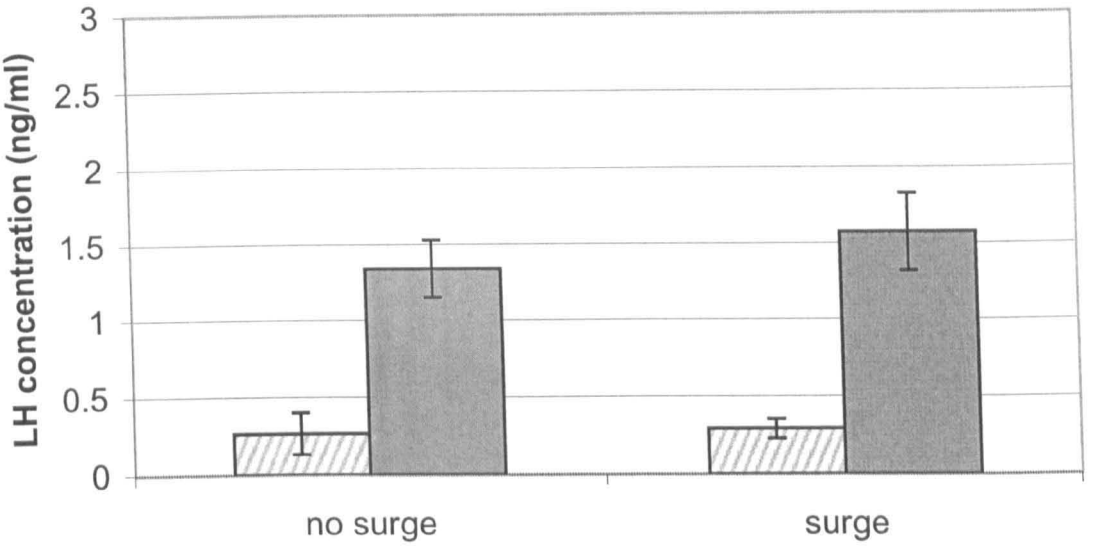


Figure 7.2 Histogram illustrating the mean (± sem) LH concentrations of MRB ewes before (grey bars) and after (black bars) that were detected (n=4) or not detected (n=4) with an LH surge between 18 and 42 hours after ram introduction

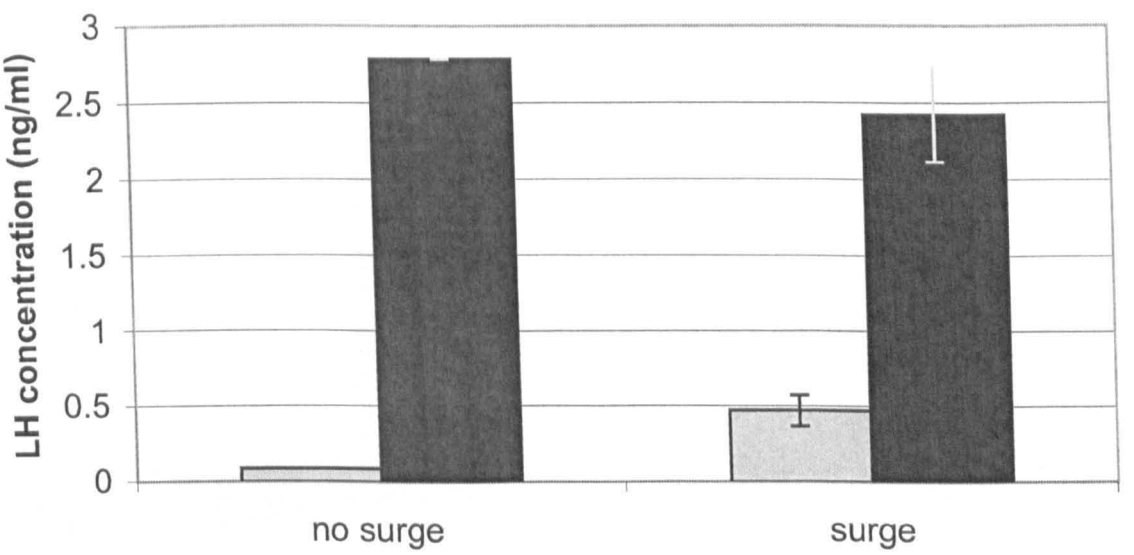


Figure 7.3 Histogram illustrating the mean (± sem) LH concentrations of MCB ewes before (grey bars) and after (black bars) that were detected (n=8) or not detected (n=1) with an LH surge between 18 and 42 hours after ram introduction

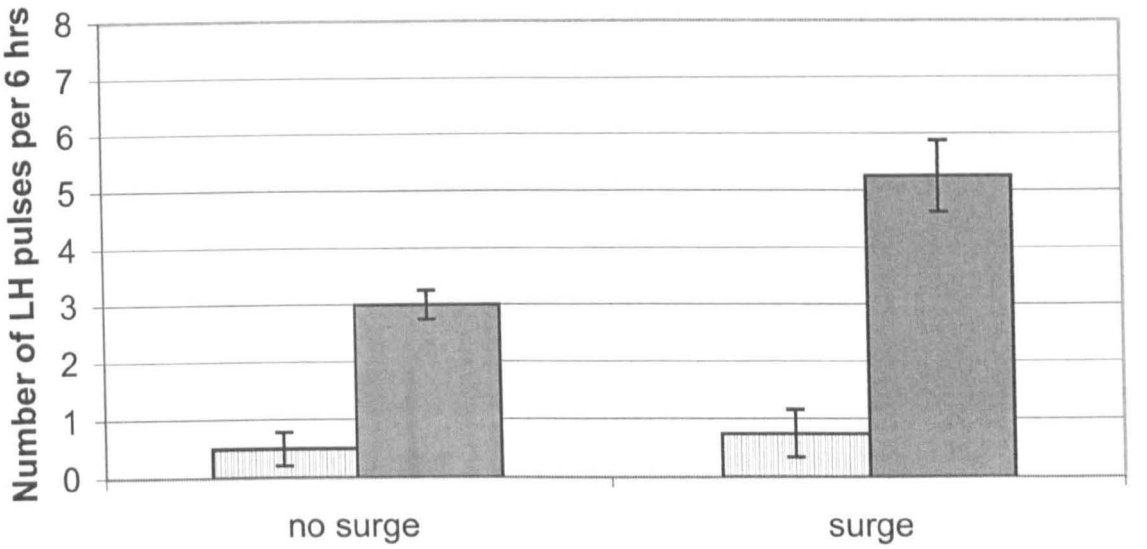


Figure 7.4 Histogram illustrating the mean (± sem) number of pulses before (grey vertical hatched bars) and after (black hatched bars) ram introduction of ram experienced ewes that were detected (n=4) or were not detected (n=4) with an LH surge between 18 and 42 hours after ram introduction

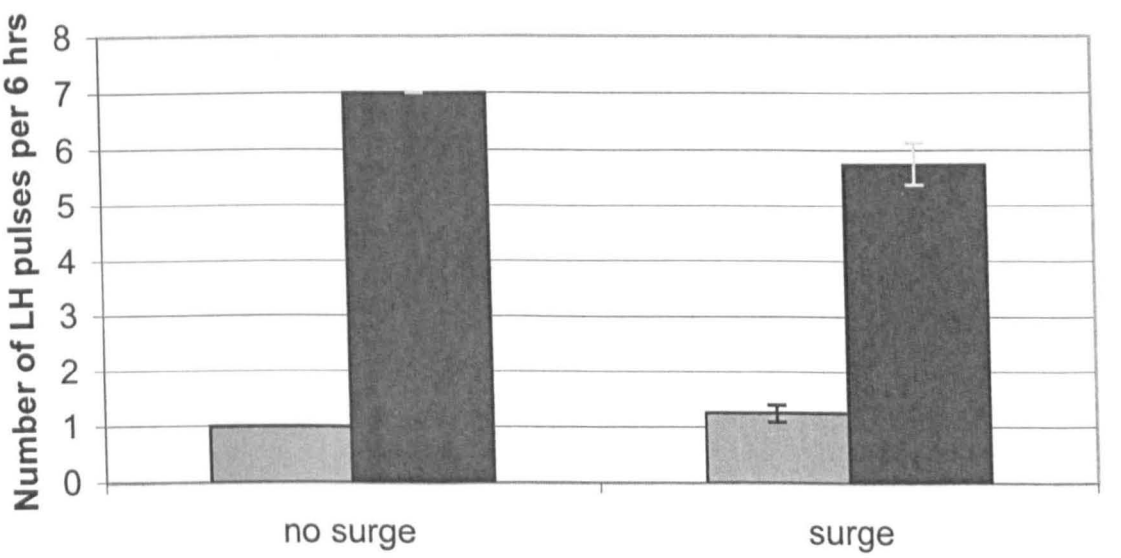


Figure 7.5 Histogram illustrating the mean (± sem) number of pulses before (grey vertical hatched bars) and after (black hatched bars) ram introduction of ram naïve ewes that were detected (n=8) or were not detected (n=1) with an LH surge between 18 and 42 hours after ram introduction.

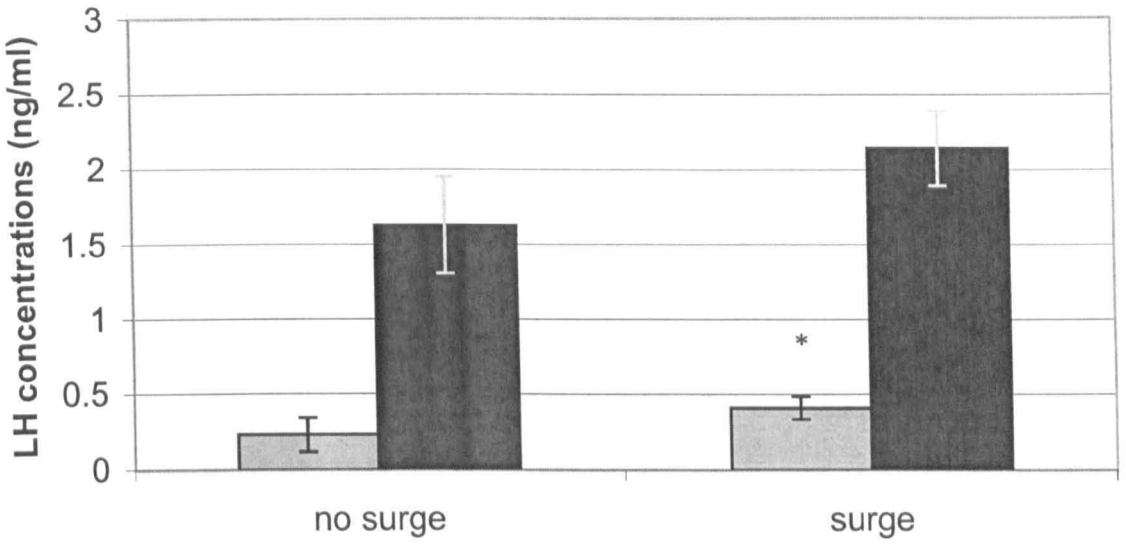
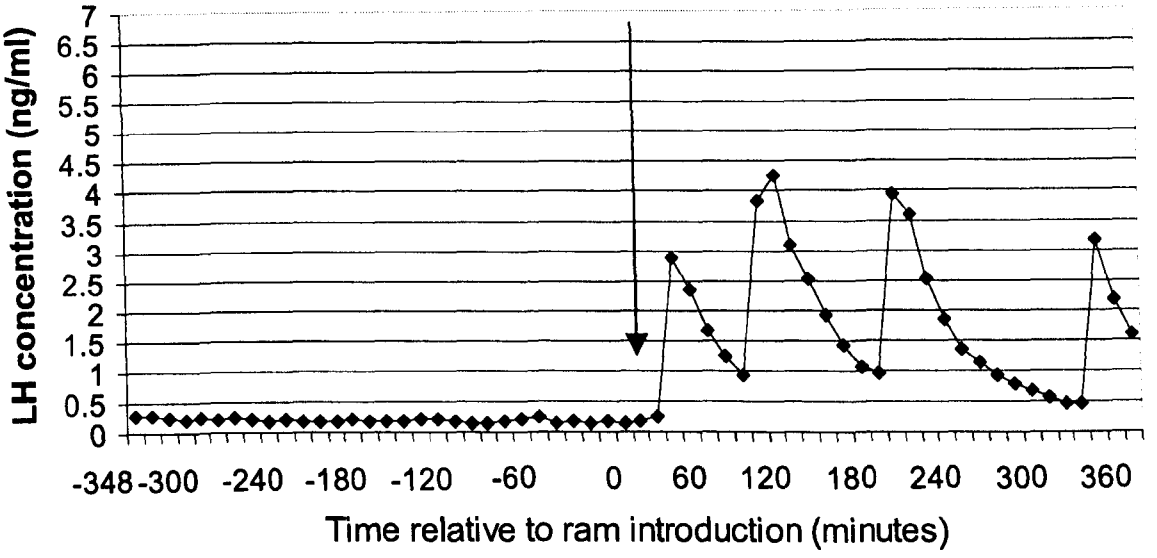


Figure 7.6 Histogram illustrating the mean (±sem) concentrations of LH during the 6-hour period before (grey bars) and after (black bars) ram introduction in ewes detected with (n=11) or without (n=5) an LH surge. Before ram introduction ewes detected with an LH surge had significantly LH concentrations over the 6-hour sampling period than ewes not detected with an LH surge (*$P<0.05$). However there was no significant difference in the mean LH concentrations after ram introduction in ewes detected with or without an LH surge ($P>0.1$).

Ewe 13



Ewe 2

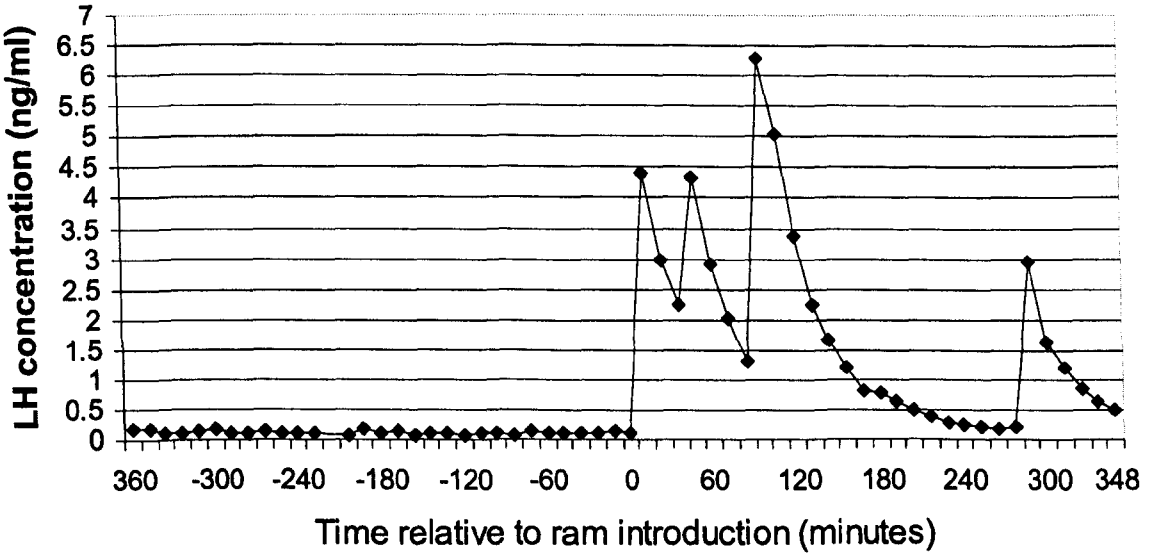
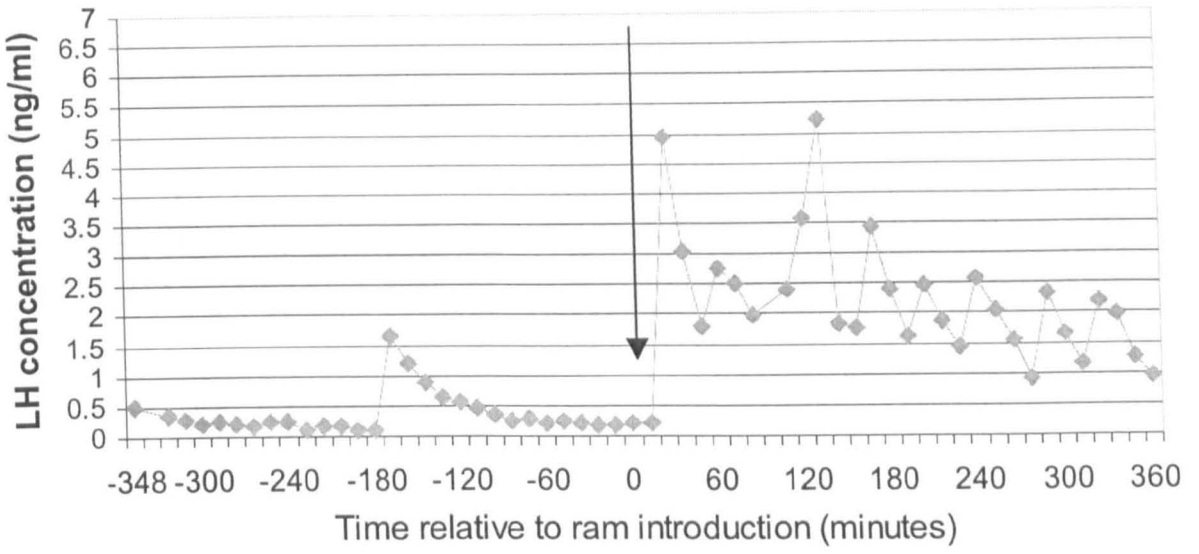


Figure 7.7 Example profiles of ram experienced ewes detected or not detected with an LH surge between 18 and 42 hours after ram introduction. Ewe 13 had an LH surge at 32 hours after ram introduction with a maximum LH concentration of 27.9ng/ml however duration could not be calculated, as the LH concentrations did not reach baseline within the sampling period. Ewe 2 was not detected with an LH surge between 18 and 42 hours after ram introduction.

Ewe 8



Ewe 17

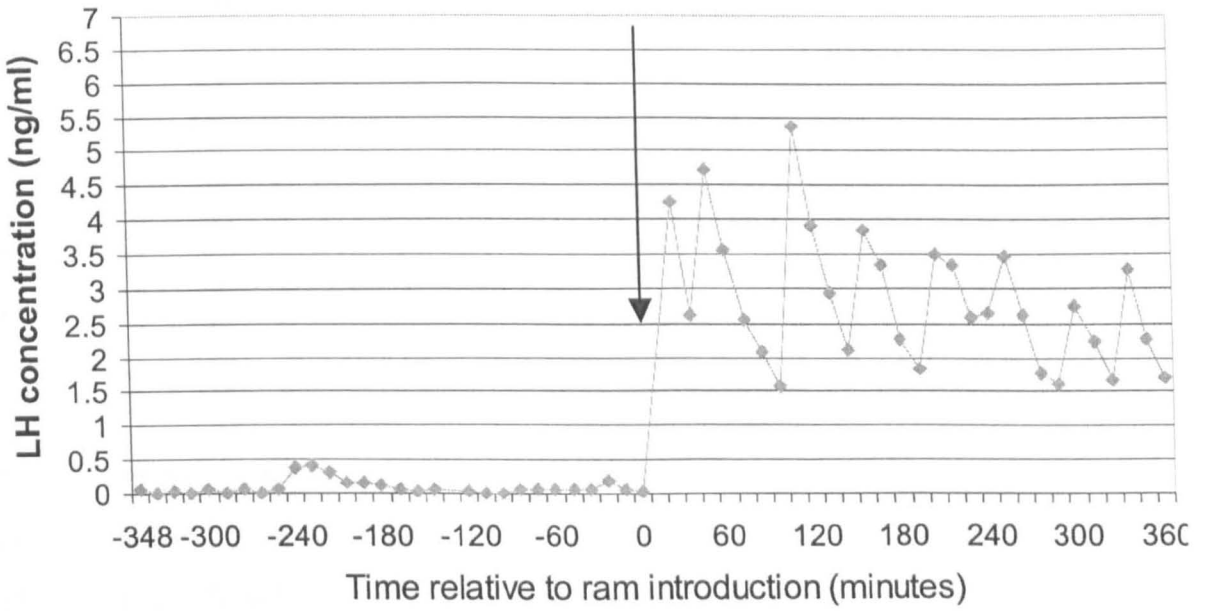


Figure 7.8 Example profiles of ram naive ewes detected or not detected with an LH surge between 18 and 42 hours after ram introduction. Ewe 17 was not detected with an LH surge between 18 and 42 hours after ram introduction. Ewe 8 had an LH surge at 22 hours after ram introduction with duration of 20 hours and maximum LH concentration of 37.10 ng/ml.

7.5 DISCUSSION

Ram introduction induced a significant increase in mean and basal LH concentrations and LH pulse frequency within both ram experienced and ram naïve ewes. There was no effect of ram introduction on LH pulse amplitude. Ram naïve ewes had a higher LH pulse frequency both before and after ram introduction than ram experienced ewes, however there was no interaction between prior experience of the ram and the magnitude of the increase in LH pulse frequency after ram introduction. There were no significant differences between ram naïve and ram experienced ewes in any other parameters of the LH response.

The significantly higher LH pulse frequency both before and after ram introduction in the ram naïve ewes was associated with a greater proportion of ewes detected with and an early onset of an LH surge. LH pulse frequency during anoestrus can be perceived as a measure of the depth of anoestrus (Martin *et al.*, 1985). For example, ovariectomised Suffolk ewes have an innately lower LH pulse frequency during anoestrus than ovariectomised Merino ewes (Martin *et al.*, 1986). Depth of anoestrus is a powerful factor modulating the ovulatory response of ewes to the ram effect (Lindsay and Signoret, 1980). Therefore it may be that the earlier LH surge following ram introduction to the ram naïve ewes is more a reflection of the endocrine state of these ewes prior to ram introduction than a difference in the endocrine response to the ram effect.

The reason for the higher pulse frequency prior to ram introduction in the ram naïve ewes is unclear. Ram exposure can stimulate puberty in pre-pubertal ewe lambs (Al-Mauly *et al.*, Knights *et al.*, 2002). However spring born lambs typically attain puberty during the autumn, aged between 25 and 35 weeks of age (Foster and Ryan, 1979). This is supported relative to the mule ewe by the findings of Al-Mauly *et al.*, (1991) where spring born mule ewe lambs isolated from ram contact reached puberty in early November. Therefore it is unlikely that the difference in pre-ram introduction pulse frequency between ram naïve and ram experienced ewes originates from ewes being pre or post pubertal at the time of ram introduction.

Continuous ram presence during the transition into anoestrus extends the breeding season (O'Callaghan *et al.*, 1994). If ewes are maintained in continuous contact with

rams this extension is reportedly lost and ewes have a pattern of seasonal transitions similar to ewes maintained in absence of rams (Riches and Watson, 1954). However in this study the rams were present for one month and removed at the end of December, which is earlier than the natural transition into the anoestrous period identified in studies conducted at a comparable latitude (6th March ± 7 days; O'Callaghan *et al.,* 1994). However maiden ewes in the first autumn of life typically have a shorter breeding season than adult ewes (Bathaei, 1996) and the transition into anoestrus is a gradual process (Karsch *et al.,* 1984a). Therefore I propose that the continuous exposure of maiden ewes to rams late during the breeding season may have affected the transition into anoestrus. I hypothesise that this may have resulted in ram-experienced ewes being at a greater depth of anoestrus when subsequently introduced to rams later during the anoestrous period.

The supplementation of ewes with concentrate feed in this experiment may explain the higher mean and basal concentrations of LH and LH pulse frequency prior to ram introduction and relative magnitude of the LH response compared to anoestrous ewes in Chapter 5. A low plane of nutrition is associated with increased responsiveness of the hypothalamic-pituitary axis to the negative effects of oestradiol (Lindsay, 1996). Changes in the plane of nutrition induces rapid changes in the endocrine milieu of rams (Review, Martin and Walkden-Brown, 1995) and evidence in goats indicates that this can markedly improve their endocrine response to the introduction of oestrous females (Walkden-Brown *et al.,* 1994). Though the role of nutrition in the responses of the ewe to the ram effect is less equivocal in sheep (Review; Walkden-Brown *et al.,* 1999) nutrition is a powerful modulator of LH concentrations (Walkden-Brown *et al.,* 1999). Furthermore Forcada *et al.,* (2002) identified that a high plane of nutrition modified the steroidgenic control of LH release during anoestrus. Therefore I propose that supplementation of the ewes in this study with concentrated feed enhanced the magnitude of all parameters of the LH response both before and after ram introduction.

In summary, the introduction of rams to maiden ewes during late anoestrus (August) induced a significant increase in basal and mean LH concentrations and LH pulse frequency in both maiden ewes with or without prior experience of the ram during the

breeding season. I had proposed that prior experience of the ram during the breeding season would enhance the endocrine response of maiden ewes to ram introduction. In contrast ram experienced maiden ewes had a lower LH pulse frequency both before and after ram introduction. I propose that the lower LH pulse frequency after ram introduction is related to the lower LH pulse frequency before ram introduction. This difference in the pre-ram exposure endocrine state may have occurred by chance or be an artifact of the time of the pre-conditioning ram exposure period relative to the cessation of the natural breeding season. There were no other positive or negative effects of prior experience of the ram during the breeding season on any other parameters of the LH response. Ewes having an LH surge had greater LH concentrations prior to ram introduction and within ram experienced ewes, a higher LH pulse frequency after ram introduction. These findings highlight the importance of the endocrine milieu prior to ram introduction in relation to the characteristics of the subsequent LH response and the successful stimulation of ewes with the ram effect.

8. COMPARISON BETWEEN A NON-PHARMACOLOGICAL METHOD OF OESTRUS SYNCHRONISATION AND ARTIFICIAL SYNCHRONISATION IN EWES ARTIFICIALLY INSEMINATED DURING THE BREEDING SEASON

8.1 ABSTRACT

Artificial insemination is a valuable tool in the sheep industry however conception rates and poor fertility with frozen semen restrict its application. Artificial progestagens have been attributed to contribute to the low conception rates. Within Norwegian insemination systems, ewes are mated to a natural oestrus with reported high conception rates and overall fertility even with frozen semen. Therefore the aim of this experiment was to compare the conception rates and litter size obtained in ewes synchronised using artificial progestagens (artificially synchronised, AS; n=55) or using the repeated ram exposure strategy developed in Chapter 3 (ram synchronised, RS; 100). On Day 50 after the first ram exposure of the RS ewes (16 days after the final ram exposure period) vasectomised rams were introduced to the RS ewes. Raddle marks were monitored over the subsequent three-day period and marked ewes were drafted off once a day and ewes were inseminated within 4 hours of drafting. The AS ewes were treated for 14 days with an intravaginal progestagen pessary and inseminated between 50 and 56 hours after sponge withdrawal. RS ewes had significantly greater conception rates than artificially synchronised ewes ($P<0.01$). Within the RS ewes, ewes marked by the vasectomised rams one cycle prior to insemination had a significantly lower conception rate than those ewes first marked by vasectomised rams on the day of insemination ($P<0.05$). Within ewes lambing to the inseminated service there was no significant difference in the mean litter size of RS and AS (1.57 ± 0.13 versus 1.58 ± 0.19, RS and AS ewes respectively). The adequate conception rates observed in this study in the RS ewes potentiates the use of the repeated ram exposure strategy developed in Chapter 3 as a non-pharmacological method of oestrus synchronisation for use in an artificial insemination programme.

8.2 INTRODUCTION

Artificial insemination (AI) is a valuable tool in the sheep industry and plays an important role in the distribution of superior genetics within UK flocks (Khalid *et al.*, 1998). However AI in sheep is more complex than within other species with conception rates comparable to natural mating only reliably possible with frozen semen through use of laparoscopic AI (Khalid *et al.*, 1998). However there are ethical concerns over the routine use of intrauterine AI (Gordon, 1997) due to the invasive nature of the procedure.

One of the simplest methods of inseminating the ewe is by cervical insemination using fresh or chilled semen where sperm is deposited in the first fold of the cervix. Conception rates using this method of artificial insemination and fresh semen range from between 65%, (Evans and Maxwell, 1987) to 82% (Donovan *et al.*, 2004) however they are extremely dependent on technician, breed and season (Gordon, 1997; Donovan *et al.*, 2004). A further complicating factor stems from a negative effect of artificial synchronisation on conception rates (Quinlaven and Robinson, 1969) proposed to be driven by reduced sperm transport through the cervix (Killeen and Cafferty, 1982) however evidence of this is equivocal (Donovan *et al.*, 2004). Furthermore when incorporated with frozen semen, cervical artificial insemination after artificial synchronisation is associated with a depression in litter size (Langford *et al.*, 1979; Oleson, 1993; Donovan *et al.*, 2004).

Conception rates to cervical artificial insemination improve when ewes are inseminated during the natural breeding season due to the physiological state of the ewe and the improved sperm production within the donor rams (Gordon, 1997). Therefore the above factors suggest there is a greater probability of improved conception rates within ewes served at a natural oestrus in the absence of artificial synchronisation. However the labour and time associated with oestrous detection in ewes maintained at pasture (Donovan *et al.*, 2004) and the natural distribution of oestrus in randomly cycling ewes are major limiting factors to the efficiency of an artificial insemination protocol using ewes mated to a natural oestrus.

Conventional application of the ram effect during anoestrus has been shown to be effective as a method of synchronising Merino ewes for artificial insemination (Corke,

1980). Within Chapter 3, I developed a strategy for synchronous mating during the breeding season using intermittent repeated exposures to vasectomised rams repeated at 17-day intervals. When entire rams were introduced for mating, 36% of ram-exposed ewes were mated during the first 72 hours of entire ram introduction with 100% of ewes mated within 8 days. This type of ram-induced synchronisation offers a condensed and predictable distribution of oestrus and thus I propose that it could be applied as a method of ewe synchronisation for an artificial insemination programme.

The aim of this experiment was to compare conception rates and litter size in ewes inseminated at a fixed time after artificial synchronisation of oestrus using conventional progestagen pessaries with ewes inseminated after synchronisation using the repeated, intermittent vasectomised ram exposure programme developed in Chapter 3.

8.3 MATERIALS AND METHODS

8.3.1 ANIMALS AND EXPERIMENTAL PROCEDURES

The study was conducted at Cockle Park Research Farm, Northumberland (55°13'N), using multiparous mule ewes (Swaledale x Bluefaced/Border Leicester) that had been previously isolated from ram contact (not within 500m). During September, ewes (n=155) were exposed to vasectomised rams (n=3) for 24 hours on Days 0 (September 7th), 17 and 34 of the experiment. Raddle marks were recorded at the end of each exposure and raddle colour was changed before the next exposure period to monitor continued or first incidence of oestrus. On Day 35 a random selection of ewes that had not been marked by the vasectomised rams during the exposure periods were allocated to Group AS (Artificially Synchronised; n=55). The remaining unmarked and marked ewes were allocated to Group RS (Ram Synchronised; n=100).

8.3.1.1 ARTIFICIALLY SYNCHRONISED EWES

AS ewes were treated for 13/14 days with an intravaginal progestagen pessary (60mg medroxyprogesterone acetate, Veramix sponge, Upjohn, UK) inserted on Day 36 (ASA; n=25) and Day 37 (ASB; n=32) of the experiment. The insertion of the intravaginal sponges was staggered to permit removal of the progestagen pessary and artificial insemination of the ewes over a 3 day period with each ewe having had a

minimum synchronisation period of 13 days. Intravaginal pessaries were removed on Day 49 (ASA; n=23), Day 50 (ASA; n=2, ASB; n=17) and Day 51 (ASB; n=15) . These ewes were drafted off from the main group and inseminated between 50-56 hours after sponge removal (Bright *et al.*, 1998) on Days 1, 2 and 3 of the insemination protocol respectively. After insemination ewes were maintained at pasture with the inseminated RS ewes. One week after insemination raddled entire rams (n=4) were introduced to detect and mate any ewes not conceiving to the inseminated service. Ewes were subsequently maintained according to conventional farm practice until lambing when date of lambing and number of lambs born were recorded.

8.3.1.2 RAM SYNCHRONISED EWES

Raddled vasectomised rams (n=2) were introduced to RS ewes on Day 50 (27th October) and remained with the ewes for four days. Raddle marks were checked on the morning of Days 51, 52 and 53 and any marked ewes were drafted off and inseminated within 4 hours of drafting on Days 1 (n=20), 2 (n=14) and 3 (n=6) of the insemination protocol respectively. After insemination ewes were maintained with the AS ewes as outlined above.

8.3.2 RAM SEMEN COLLECTION

Semen was collected using an artificial vagina (Dan Fawcett Consulting Ltd, Cumbria) and immediately placed in a thermo stable water bath (30°C) prior to assessment. Each semen sample was evaluated by the same operator to provide continuity in the subjective assessment of density and motility. After collection, the volume of the ejaculate was recorded and scored for motility and density. Wave motion was assessed by placing a small droplet of semen on a microscope slide and evaluated using the scale in Table 8.1 at a magnification of (x 10). Density of the sample was assessed subjectively using the scale in Table 8.2. The number of ewes to be inseminated with each sample was calculated using the equation below to estimate the total live number of sperm. The ejaculate was maintained in a thermo stable water bath (30°C) diluted and used within 1 hour of collection. All rams used were of the same breed (Texel) and multiple ejaculates were taken from each ram to inseminate the ewes in heat on that day. Each ejaculate was independently assessed and its

suitability verified prior to use in the insemination procedure and no more than three ejaculates were taken from any ram.

Equation for calculation of numbers of ewes to be inseminated per ejaculate

Number of live sperm in ejaculate = Volume x Number of sperm (as derived from the density score) x Motility

Based on each ewe requiring 0.1×10^9 sperm (Dan Fawcett, personal communication) the total number of live sperm calculated above is divided by 0.1×10^9 sperm to give the number of ewes that can be inseminated with that ejaculate.

Dilution of semen

Semen was diluted according to the motility score of the semen. For example, if the sample was scored with a 5 for motility it was diluted 1 part semen to 5 parts UHT milk.

The numbers of AS and RS ewes inseminated each day were balanced as far as possible (within the numbers available) to minimise any bias effect of ram fecundity on any one group. For each ewe the AI pipette was loaded with 0.4ml of diluted semen and withdrawn from the tip by drawing up 0.2ml of air. The pipette was held in cotton wool to provide thermal stability between preparation and use. Between inseminations the AI pipette was wiped using dry paper towel.

Table 8.1 Scoring system for wave motion – (Bright *et al.*, 1998)

Score	Class Description
5	Very good dense, very rapidly moving waves. Individual sperm cannot be observed. More than 90% of the sperm are active.
4	Good Vigorous movement, however waves and eddies are not as rapid as those in score 5. About 70 to 90% of the sperm are active.
3	Only small, slow moving waves. Individual sperm may be observed. About 40 to 65% of sperm are active.
2	Poor. No wave motion forming, but some movement of sperm is visible. About 20 to 40% of sperm are active.
1	Very poor About 10% of sperm active. Possible to observe slight 'flickering' of sperm with poor motility.
0	Dead. No movement apparent

Table 8.2 Concentration of ram semen assessed for consistency - (Bright *et al.*, 1998)

Density	Number of sperm per ml
Thick creamy	4.5×10^9
Creamy	3.5×10^9
Thin creamy	3.0×10^9
Milky	2.0×10^9
Cloudy	0.7×10^9
Clear (watery)	Insignificant

8.3.3 INSEMINATION PROCEDURE

The ewe was restrained using the "over the rail" technique (Salamon, 1976) and held firmly during the insemination procedure. The vulva was cleaned using warm water and paper towel. A well-lubricated speculum was inserted vertically into the entrance to the vagina, gently rotated 90° and opened to show the vaginal canal. The entrance to the cervix was viewed using a pen torch and any unusual observations or excessive vaginal mucus were recorded. The tip of the AI pipette was then inserted into the first folds of the cervix, the diluted semen deposited and the speculum closed and retracted. The time and date of insemination, ram and ram ejaculate were recorded for each ewe. A single inseminator conducted all inseminations. Between inseminations the speculum was disinfected using a non-spermicidal disinfectant (Decon 90; Dan Fawcett Consulting Ltd, Cumbria) and wiped dry.

8.3.4 DATA ANALYSIS

Data relating to conception rates was analysed by binary logistic regression (Minitab 1.7) with treatment, factors relating to the pre-insemination procedure (ram synchronised ewes: marked by rams during the 1st, 2nd or 3rd exposure period; artificially synchronised ewes: date of sponge insertion) and parameters relating to insemination procedure included in the regression analysis. The effect of treatment and the above parameters related to the pre-insemination and insemination procedure on the numbers of ewes having single twin or triplet lambs was analysed by ordinal logistic regression.

8.4 RESULTS

Ram synchronised ewes had a significantly greater conception rate than artificially synchronised ewes (60% versus 21%, RS and AS ewes respectively; $P<0.01$). Date and time of insemination, date of sponge insertion, ram used and ejaculate number had no significant effect on conception rates. Within the RS ewes, ewes marked by the vasectomised rams one cycle prior to insemination had a significantly lower conception rate than those ewes first marked by vasectomised rams on the day of insemination (44% versus 92%; $P<0.05$). There was no significant effect of being marked by the vasectomised rams two cycles prior to mating (n=3) on conception rates (33% versus 62%; $P>0.1$).

Within ewes lambing to the inseminated service there was no significant difference in the numbers of RS and AS ewes having single (48% versus 50%) twin (48% versus 42%) or triplet (4% versus 8%) lambs. This was also reflected by the similar mean litter size (1.57 ± 0.13 versus 1.58 ± 0.19, RS and AS ewes respectively). Furthermore there was no effect of date and time of insemination, date of sponge insertion, whether ewes had been previously marked by the vasectomised rams, ram used or ejaculate number on the proportion of ewes having single, twin or triplet lambs.

There was no significant difference between treatments in the numbers of ewes that were barren, aborted or died during the experiment (4% versus 0%; RS and AS ewes respectively).

8.5 DISCUSSION

The higher conception rate within ram-synchronised ewes is in agreement with previous studies where insemination of ewes to a natural rather than artificially induced oestrus resulted in comparably higher conception rates (Robinson and Moore, 1967). However the conception rates of both artificially and ram synchronised ewes in this study are markedly lower than previously reported with fresh semen in other studies (artificial, 21% versus 52% Maxwell, 1986, 70% Donovan *et al.*, 2004; natural, 60% versus 70%; Jonmundsson, 1986, 82% Donovan *et al.*, 2004).

A considerable number of factors affect fertility to artificial insemination including inseminator, ram, breed, stress and progestagen type (Oleson, 1993). However in this study, parameters relating to the insemination procedure were the same for all ewes and regression analysis found no significant effect of any of the parameters on conception rates to the inseminated service. With respect to the ewes mated to a natural oestrus, the double drafting procedure used by Donovan *et al.*, (2004) is likely to be a preponderant factor in the greater conception rates in their study of ewes inseminated to a natural oestrus. This type of double drafting procedure allows detection of ewes at an optimal time of insemination relative to detection of oestrus. Oleson (1993) identified an optimal time of cervical insemination with frozen semen at 15-20 hours after detection of oestrus. Therefore by drafting ewes off once per day a proportion of ewes will be inseminated before or after the optimal time relative to ovulation.

With respect to the artificially synchronised ewes, the time of AI relative to sponge withdrawal in this study (50-56 hrs) is marginally earlier than that recommended by Maxwell, (1984) at 55-56 hrs after sponge withdrawal. However Donovan *et al.*, (2001) found no significant effect of a 6-hour difference in conception rates of ewes cervically inseminated with frozen semen. The use of vasectomised rams to detect ewes in oestrus after sponge withdrawal markedly improves conception rates to artificial insemination. Therefore the insemination of ewes at a fixed time rather than in combination with oestrous detection using vasectomised rams is likely to have contributed to the lower conception rates observed in this study. Furthermore the type of progestagen used for oestrus synchronisation has been shown to markedly affect conception rates due to the distribution of oestrus after sponge withdrawal (Pearce *et*

al. 1984). Gordon, (1983) compared conception rates in ewes synchronised by intravaginal pessaries treated with medroxyprogesterone acetate (MAP) or flurogestone acetate (FGA) when mated to natural service and artificial insemination. Though there was no comparative difference in conception rates in ewes mated by the rams, there was a significant advantage of using FGA when ewes were artificially inseminated (Gordon, 1983). In this study ewes were synchronised using a 13/14-day MAP protocol in contrast to Donovan *et al.*, (2004) where ewes were synchronised using FGA intravaginal pessaries. Therefore, I propose that the use of MAP sponges and the absence of vasectomised rams to detect oestrus in the artificially synchronised ewes were two of the factors contributing to the depressed conception rates in this study.

The comparative depression of conception rates in both ewes inseminated to a natural and artificially induced oestrus may also be directly related to my inexperience in cervical artificial insemination. Inseminator skill is a major determining factor in conception rates and litter size in artificially inseminated ewes (Oleson, 1993) therefore, I propose that my relative inexperience in this technology contributed to the observed conception rates in this study.

Ram synchronised ewes marked by vasectomised rams during the oestrous cycle prior to insemination had significantly lower conception rates than ewes marked by vasectomised rams for the first time immediately prior to insemination. This is in contrast to the findings by Berg and Aaamdal (1991) where ewes inseminated to the second oestrus of the breeding season had higher conception rates compared to ewes inseminated to the first oestrus. Expression of behavioural oestrus requires a period of progesterone priming (Robinson, 1954) thus the first ovulation of the breeding season is typically silent (Karsch, 1984). The ram-synchronised ewes were exposed periodically to vasectomised rams every 17 days from the transition into the breeding season. Therefore ewes marked by vasectomised rams during the oestrous cycle prior to insemination are likely to have had a full oestrous cycle either stimulated coincidentally by the advancing photoperiod or by the ram exposure approximately one oestrous cycle length prior to insemination. Ram presence during a natural or artificially induced follicular phase advances the onset and reduces the duration of oestrus (natural: Fletcher and Lindsay, 1971; artificial: Romano *et al.*, 2000; 2001)

and advances the LH surge and ovulation (artificial: Maxwell, 1984, Lucidi *et al.*, 2001; natural: Cahill *et al.*, 1974; Lindsay *et al.* 1975). Therefore, I propose that the repetitive exposure of receptive and thus marked ewes to rams at the interval of approximately one cycle length is likely to cause a gradual shift in the time of oestrus and ovulation. Asynchrony between ovulation and insemination is one of the most common causes of reproductive wastage in artificially inseminated ewes due to ageing of either the sperm or oocyte (Lucidi *et al.*, 2001). Delayed insemination relative to the time of ovulation is typically associated with fertilisation of aged oocytes and reduced embryo viability and survival rates in a number of species (sheep; McEvoy *et al.*, 1995, cattle; Dalton *et al.*, 2001). Therefore, I propose that the depression in conception rates in ewes marked during the oestrous cycle prior to insemination was due to asynchrony between ovulation and insemination induced by an effect of the rams during the final exposure period 17 days prior to insemination that was further aggravated by vasectomised ram introduction for oestrous detection the day prior to insemination. This theory is supported by the exceptionally low conception rates of fence-line ram exposed ewes mated during the first 24 hours of ram introduction in Chapter 3 that was curtailed in subsequent studies by entire ram introduction at 16 rather than 17 days after the final ram exposure period.

The absence of any significant difference in litter size between ram synchronised and artificially synchronised ewes is in agreement with findings of Donovan *et al.*, (2004) and Mitchell *et al.*, (1999) where there was no significant difference in litter size between ewes mated to a natural or artificially induced oestrus. Oleson (1993) estimated a depression in litter size of 0.2-0.4 lambs in ewes artificially inseminated compared to natural service. Our results concur with this estimation as from Chapter 3 the ewes naturally mated during the same autumn had a litter size ranging from 1.8 to 2.2 lambs per ewe. However Donovan *et al.*, (2004) identified a significantly greater depression in litter size in ewes inseminated with frozen-thawed semen and inseminated to an artificially synchronised oestrus than in ewes inseminated to a natural oestrus. Therefore as the method of ram synchronisation in this study permits insemination to a natural service, it may be that it has potential applications to the improvement of litter size in ewes inseminated with frozen-thawed semen.

In summary, short-term, repeated exposure of ewes to rams during the transition into the breeding season compacted the oestrous activity of 40% of ram-synchronised ewes over a three-day insemination period. The conception rate of these ram-synchronised ewes was significantly greater than ewes artificially synchronised by a 13-day medroxyprogesterone acetate treatment. However the particularly low conception rates are likely to be a product of a less than optimal time of insemination of these ewes relative to progestagen withdrawal.

PAGE

NUMBERING

AS ORIGINAL

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8.1 ABSTRACT

Artificial insemination is a valuable tool in the sheep industry however conception rates and poor fertility with frozen semen restrict its application. Artificial progestagens have been attributed to contribute to the low conception rates. Within Norwegian insemination systems, ewes are mated to a natural oestrus with reported high conception rates and overall fertility even with frozen semen. Therefore the aim of this experiment was to compare the conception rates and litter size obtained in ewes synchronised using artificial progestagens (artificially synchronised, AS; n=55) or using the repeated ram exposure strategy developed in Chapter 3 (ram synchronised, RS; 100). On Day 50 after the first ram exposure of the RS ewes (16 days after the final ram exposure period) vasectomised rams were introduced to the RS ewes. Raddle marks were monitored over the subsequent three-day period and marked ewes were drafted off once a day and ewes were inseminated within 4 hours of drafting. The AS ewes were treated for 14 days with an intravaginal progestagen pessary and inseminated between 50 and 56 hours after sponge withdrawal. RS ewes had significantly greater conception rates than artificially synchronised ewes ($P<0.01$). Within the RS ewes, ewes marked by the vasectomised rams one cycle prior to insemination had a significantly lower conception rate than those ewes first marked by vasectomised rams on the day of insemination ($P<0.05$). Within ewes lambing to the inseminated service there was no significant difference in the mean litter size of RS and AS (1.57 ± 0.13 versus 1.58 ± 0.19, RS and AS ewes respectively). The adequate conception rates observed in this study in the RS ewes potentiates the use of the repeated ram exposure strategy developed in Chapter 3 as a non-pharmacological method of oestrus synchronisation for use in an artificial insemination programme.

8.2 INTRODUCTION

Artificial insemination (AI) is a valuable tool in the sheep industry and plays an important role in the distribution of superior genetics within UK flocks (Khalid *et al.*, 1998). However AI in sheep is more complex than within other species with conception rates comparable to natural mating only reliably possible with frozen semen through use of laparoscopic AI (Khalid *et al.*, 1998). However there are ethical concerns over the routine use of intrauterine AI (Gordon, 1997) due to the invasive nature of the procedure.

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minimum synchronisation period of 13 days. Intravaginal pessaries were removed on Day 49 (ASA; n=23), Day 50 (ASA; n=2, ASB; n=17) and Day 51 (ASB; n=15) . These ewes were drafted off from the main group and inseminated between 50-56 hours after sponge removal (Bright *et al.,* 1998) on Days 1, 2 and 3 of the insemination protocol respectively. After insemination ewes were maintained at pasture with the inseminated RS ewes. One week after insemination raddled entire rams (n=4) were introduced to detect and mate any ewes not conceiving to the inseminated service. Ewes were subsequently maintained according to conventional farm practice until lambing when date of lambing and number of lambs born were recorded.

8.3.1.2 RAM SYNCHRONISED EWES

Raddled vasectomised rams (n=2) were introduced to RS ewes on Day 50 (27th October) and remained with the ewes for four days. Raddle marks were checked on the morning of Days 51, 52 and 53 and any marked ewes were drafted off and inseminated within 4 hours of drafting on Days 1 (n=20), 2 (n=14) and 3 (n=6) of the insemination protocol respectively. After insemination ewes were maintained with the AS ewes as outlined above.

8.3.2 RAM SEMEN COLLECTION

Semen was collected using an artificial vagina (Dan Fawcett Consulting Ltd, Cumbria) and immediately placed in a thermo stable water bath (30°C) prior to assessment. Each semen sample was evaluated by the same operator to provide continuity in the subjective assessment of density and motility. After collection, the volume of the ejaculate was recorded and scored for motility and density. Wave motion was assessed by placing a small droplet of semen on a microscope slide and evaluated using the scale in Table 8.1 at a magnification of (x 10). Density of the sample was assessed subjectively using the scale in Table 8.2. The number of ewes to be inseminated with each sample was calculated using the equation below to estimate the total live number of sperm. The ejaculate was maintained in a thermo stable water bath (30°C) diluted and used within 1 hour of collection. All rams used were of the same breed (Texel) and multiple ejaculates were taken from each ram to inseminate the ewes in heat on that day. Each ejaculate was independently assessed and its

suitability verified prior to use in the insemination procedure and no more than three ejaculates were taken from any ram.

Equation for calculation of numbers of ewes to be inseminated per ejaculate

Number of live sperm in ejaculate = Volume x Number of sperm (as derived from the density score) x Motility

Based on each ewe requiring 0.1×10^9 sperm (Dan Fawcett, personal communication) the total number of live sperm calculated above is divided by 0.1×10^9 sperm to give the number of ewes that can be inseminated with that ejaculate.

Dilution of semen

Semen was diluted according to the motility score of the semen. For example, if the sample was scored with a 5 for motility it was diluted 1 part semen to 5 parts UHT milk.

The numbers of AS and RS ewes inseminated each day were balanced as far as possible (within the numbers available) to minimise any bias effect of ram fecundity on any one group. For each ewe the AI pipette was loaded with 0.4ml of diluted semen and withdrawn from the tip by drawing up 0.2ml of air. The pipette was held in cotton wool to provide thermal stability between preparation and use. Between inseminations the AI pipette was wiped using dry paper towel.

Table 8.1 Scoring system for wave motion – (Bright *et al.*, 1998)

Score	Class Description
5	Very good dense, very rapidly moving waves. Individual sperm cannot be observed. More than 90% of the sperm are active.
4	Good Vigorous movement, however waves and eddies are not as rapid as those in score 5. About 70 to 90% of the sperm are active.
3	Only small, slow moving waves. Individual sperm may be observed. About 40 to 65% of sperm are active.
2	Poor. No wave motion forming, but some movement of sperm is visible. About 20 to 40% of sperm are active.
1	Very poor About 10% of sperm active. Possible to observe slight 'flickering' of sperm with poor motility.
0	Dead. No movement apparent

Table 8.2 Concentration of ram semen assessed for consistency - (Bright *et al.*, 1998)

Density	Number of sperm per ml
Thick creamy	4.5×10^9
Creamy	3.5×10^9
Thin creamy	3.0×10^9
Milky	2.0×10^9
Cloudy	0.7×10^9
Clear (watery)	Insignificant

8.3.3 INSEMINATION PROCEDURE

The ewe was restrained using the "over the rail" technique (Salamon, 1976) and held firmly during the insemination procedure. The vulva was cleaned using warm water and paper towel. A well-lubricated speculum was inserted vertically into the entrance to the vagina, gently rotated 90° and opened to show the vaginal canal. The entrance to the cervix was viewed using a pen torch and any unusual observations or excessive vaginal mucus were recorded. The tip of the AI pipette was then inserted into the first folds of the cervix, the diluted semen deposited and the speculum closed and retracted. The time and date of insemination, ram and ram ejaculate were recorded for each ewe. A single inseminator conducted all inseminations. Between inseminations the speculum was disinfected using a non-spermicidal disinfectant (Decon 90; Dan Fawcett Consulting Ltd, Cumbria) and wiped dry.

8.3.4 DATA ANALYSIS

Data relating to conception rates was analysed by binary logistic regression (Minitab 1.7) with treatment, factors relating to the pre-insemination procedure (ram synchronised ewes: marked by rams during the 1st, 2nd or 3rd exposure period; artificially synchronised ewes: date of sponge insertion) and parameters relating to insemination procedure included in the regression analysis. The effect of treatment and the above parameters related to the pre-insemination and insemination procedure on the numbers of ewes having single twin or triplet lambs was analysed by ordinal logistic regression.

8.4 RESULTS

Ram synchronised ewes had a significantly greater conception rate than artificially synchronised ewes (60% versus 21%, RS and AS ewes respectively; $P<0.01$). Date and time of insemination, date of sponge insertion, ram used and ejaculate number had no significant effect on conception rates. Within the RS ewes, ewes marked by the vasectomised rams one cycle prior to insemination had a significantly lower conception rate than those ewes first marked by vasectomised rams on the day of insemination (44% versus 92%; $P<0.05$). There was no significant effect of being marked by the vasectomised rams two cycles prior to mating ($n=3$) on conception rates (33% versus 62%; $P>0.1$).

Within ewes lambing to the inseminated service there was no significant difference in the numbers of RS and AS ewes having single (48% versus 50%) twin (48% versus 42%) or triplet (4% versus 8%) lambs. This was also reflected by the similar mean litter size (1.57 ± 0.13 versus 1.58 ± 0.19, RS and AS ewes respectively). Furthermore there was no effect of date and time of insemination, date of sponge insertion, whether ewes had been previously marked by the vasectomised rams, ram used or ejaculate number on the proportion of ewes having single, twin or triplet lambs.

There was no significant difference between treatments in the numbers of ewes that were barren, aborted or died during the experiment (4% versus 0%; RS and AS ewes respectively).

8.5 DISCUSSION

The higher conception rate within ram-synchronised ewes is in agreement with previous studies where insemination of ewes to a natural rather than artificially induced oestrus resulted in comparably higher conception rates (Robinson and Moore, 1967). However the conception rates of both artificially and ram synchronised ewes in this study are markedly lower than previously reported with fresh semen in other studies (artificial, 21% versus 52% Maxwell, 1986, 70% Donovan *et al.*, 2004; natural, 60% versus 70%; Jonmundsson, 1986, 82% Donovan *et al.*, 2004).

A considerable number of factors affect fertility to artificial insemination including inseminator, ram, breed, stress and progestagen type (Oleson, 1993). However in this study, parameters relating to the insemination procedure were the same for all ewes and regression analysis found no significant effect of any of the parameters on conception rates to the inseminated service. With respect to the ewes mated to a natural oestrus, the double drafting procedure used by Donovan *et al.*, (2004) is likely to be a preponderant factor in the greater conception rates in their study of ewes inseminated to a natural oestrus. This type of double drafting procedure allows detection of ewes at an optimal time of insemination relative to detection of oestrus. Oleson (1993) identified an optimal time of cervical insemination with frozen semen at 15-20 hours after detection of oestrus. Therefore by drafting ewes off once per day a proportion of ewes will be inseminated before or after the optimal time relative to ovulation.

With respect to the artificially synchronised ewes, the time of AI relative to sponge withdrawal in this study (50-56 hrs) is marginally earlier than that recommended by Maxwell, (1984) at 55-56 hrs after sponge withdrawal. However Donovan *et al.*, (2001) found no significant effect of a 6-hour difference in conception rates of ewes cervically inseminated with frozen semen. The use of vasectomised rams to detect ewes in oestrus after sponge withdrawal markedly improves conception rates to artificial insemination. Therefore the insemination of ewes at a fixed time rather than in combination with oestrous detection using vasectomised rams is likely to have contributed to the lower conception rates observed in this study. Furthermore the type of progestagen used for oestrus synchronisation has been shown to markedly affect conception rates due to the distribution of oestrus after sponge withdrawal (Pearce *et*

al. 1984). Gordon, (1983) compared conception rates in ewes synchronised by intravaginal pessaries treated with medroxyprogesterone acetate (MAP) or flurogestone acetate (FGA) when mated to natural service and artificial insemination. Though there was no comparative difference in conception rates in ewes mated by the rams, there was a significant advantage of using FGA when ewes were artificially inseminated (Gordon, 1983). In this study ewes were synchronised using a 13/14-day MAP protocol in contrast to Donovan *et al.,* (2004) where ewes were synchronised using FGA intravaginal pessaries. Therefore, I propose that the use of MAP sponges and the absence of vasectomised rams to detect oestrus in the artificially synchronised ewes were two of the factors contributing to the depressed conception rates in this study.

The comparative depression of conception rates in both ewes inseminated to a natural and artificially induced oestrus may also be directly related to my inexperience in cervical artificial insemination. Inseminator skill is a major determining factor in conception rates and litter size in artificially inseminated ewes (Oleson, 1993) therefore, I propose that my relative inexperience in this technology contributed to the observed conception rates in this study.

Ram synchronised ewes marked by vasectomised rams during the oestrous cycle prior to insemination had significantly lower conception rates than ewes marked by vasectomised rams for the first time immediately prior to insemination. This is in contrast to the findings by Berg and Aaamdal (1991) where ewes inseminated to the second oestrus of the breeding season had higher conception rates compared to ewes inseminated to the first oestrus. Expression of behavioural oestrus requires a period of progesterone priming (Robinson, 1954) thus the first ovulation of the breeding season is typically silent (Karsch, 1984). The ram-synchronised ewes were exposed periodically to vasectomised rams every 17 days from the transition into the breeding season. Therefore ewes marked by vasectomised rams during the oestrous cycle prior to insemination are likely to have had a full oestrous cycle either stimulated coincidentally by the advancing photoperiod or by the ram exposure approximately one oestrous cycle length prior to insemination. Ram presence during a natural or artificially induced follicular phase advances the onset and reduces the duration of oestrus (natural: Fletcher and Lindsay, 1971; artificial: Romano *et al.,* 2000; 2001)

and advances the LH surge and ovulation (artificial: Maxwell, 1984, Lucidi *et al.*, 2001; natural: Cahill *et al.*, 1974; Lindsay *et al.* 1975). Therefore, I propose that the repetitive exposure of receptive and thus marked ewes to rams at the interval of approximately one cycle length is likely to cause a gradual shift in the time of oestrus and ovulation. Asynchrony between ovulation and insemination is one of the most common causes of reproductive wastage in artificially inseminated ewes due to ageing of either the sperm or oocyte (Lucidi *et al.*, 2001). Delayed insemination relative to the time of ovulation is typically associated with fertilisation of aged oocytes and reduced embryo viability and survival rates in a number of species (sheep; McEvoy *et al.*, 1995, cattle; Dalton *et al.*, 2001). Therefore, I propose that the depression in conception rates in ewes marked during the oestrous cycle prior to insemination was due to asynchrony between ovulation and insemination induced by an effect of the rams during the final exposure period 17 days prior to insemination that was further aggravated by vasectomised ram introduction for oestrous detection the day prior to insemination. This theory is supported by the exceptionally low conception rates of fence-line ram exposed ewes mated during the first 24 hours of ram introduction in Chapter 3 that was curtailed in subsequent studies by entire ram introduction at 16 rather than 17 days after the final ram exposure period.

The absence of any significant difference in litter size between ram synchronised and artificially synchronised ewes is in agreement with findings of Donovan *et al.*, (2004) and Mitchell *et al.*, (1999) where there was no significant difference in litter size between ewes mated to a natural or artificially induced oestrus. Oleson (1993) estimated a depression in litter size of 0.2-0.4 lambs in ewes artificially inseminated compared to natural service. Our results concur with this estimation as from Chapter 3 the ewes naturally mated during the same autumn had a litter size ranging from 1.8 to 2.2 lambs per ewe. However Donovan *et al.*, (2004) identified a significantly greater depression in litter size in ewes inseminated with frozen-thawed semen and inseminated to an artificially synchronised oestrus than in ewes inseminated to a natural oestrus. Therefore as the method of ram synchronisation in this study permits insemination to a natural service, it may be that it has potential applications to the improvement of litter size in ewes inseminated with frozen-thawed semen.

In summary, short-term, repeated exposure of ewes to rams during the transition into the breeding season compacted the oestrous activity of 40% of ram-synchronised ewes over a three-day insemination period. The conception rate of these ram-synchronised ewes was significantly greater than ewes artificially synchronised by a 13-day medroxyprogesterone acetate treatment. However the particularly low conception rates are likely to be a product of a less than optimal time of insemination of these ewes relative to progestagen withdrawal.

9. INVESTIGATION INTO THE EFFECT OF RAM EXPOSURE TOWARDS THE END OF AN ARTIFICIAL SYNCHRONISATION PROTOCOL ON EWE FERTILITY AT THE SYNCHRONISED OESTRUS

9.1 ABSTRACT

Application of the "ram effect" to synchronisation protocols has focused around ram exposure post sponge removal but relatively little is known about the effect of ram exposure prior to sponge removal. Within a previous study, ewes were exposed to rams during the last three days of a progestagen synchronisation protocol and this resulted in a rapid increase in LH concentrations and an earlier LH surge (Evans *et al.*, 2004). However this response was accompanied by a depression in conception rates. It was speculated that this was due to an impact of the ram exposure on follicle development and resultant asynchrony between time of ovulation and mating when rams were introduced at 48 hours post sponge removal. The aim of this experiment was to further investigate the impact of exposure to rams during the last three days of a progestagen synchronisation protocol in conjunction with ram introduction for mating at 24 hours post sponge removal. During October, 133 mule ewes underwent a 14 day synchronisation protocol commencing on Day 0. Ewes were divided into control (SC, n=67) or ram-exposed (SR, n=66) groups and on Day 11 rams were introduced to SR ewes and remained with ewes until sponge withdrawal. SC ewes remained in continued isolation from rams prior to mating. On Day 14 at sponge withdrawal, rams were removed from SR ewes and SR and SC ewes were mixed. Raddled entire rams (n=14) were introduced for mating at 24 hours post sponge removal and raddle marks were checked every 4 hours from 28-72 hours post sponge removal. There was no difference between groups in the numbers of ewes mated (97% versus 97%) or lambing to the first service (74% versus 77%). However more SR ewes yielded single lambs (39% versus 15%; P=0.007) and this led to an overall depression in litter size (1.76 ± 0.10 versus 2.10 ± 0.10; P<0.01). The similar numbers of SC and SR ewes mated and conceiving to the synchronised service indicates improved synchrony between ovulation and mating when rams were introduced at 24 hours post sponge removal. However, I hypothesise that ram introduction at 24 hours antagonised the effects of the ram exposure prior to progestagen withdrawal, resulting

in timing of the LH surge when a limited number of follicles were capable of responding, thus reducing ovulation rate and litter size.

9.2 INTRODUCTION

Intravaginal progestagen sponges are commonly used to synchronise the oestrous cycles of ewes during both the breeding and non-breeding season (Robinson, 1974). The sponge mimics the action of a corpus luteum through provision of an artificial source of progesterone sufficient to suppress gonadotrophin production. Removal of the sponge removes the progestagen block and induces synchronous re-instatement of gonadotrophin release and subsequent ovulation in treated ewes.

Exposure of previously isolated anoestrous ewes to a ram induces an instantaneous increase in pulse frequency of luteinising hormone (LH) (Martin *et al.*, 1986). The majority of research investigating the interaction between exposure to rams and controlled breeding programmes has focused on the influence of the ram after removal of the artificial progestagen (Lewis *et al.*, 1974) and have been undertaken predominantly during anoestrus (Romano *et al.*, 2001). Several studies have identified an earlier onset of oestrus and shorter duration of oestrus as a result of continuous ram presence post sponge removal (Maxwell, 1986; Romano *et al.*, 2000; 2001)

Previous work has shown that ewes under the influence of progesterone show an increase in LH pulse frequency in response to ram introduction (Martin *et al.*, 1983b). The persistent endocrinological ability of the ewe to respond to the ram, even under the influence of the artificial progestagen, denotes a possible novel opportunity to influence follicle development prior to sponge withdrawal. Previous work in cattle has shown that removal of the progestagen block at the end of the synchronisation protocol does not reliably result in a synchronous onset of ovulation due to varying stages of follicle development at sponge removal (Roche *et al.*, 1999). Similar observations have been made in sheep synchronised during the breeding season, due to an impact of stage of the oestrous cycle at sponge insertion on follicle size at sponge withdrawal (Leyva *et al.*, 1998).

We had originally hypothesised that a ram-induced increase in LH prior to sponge withdrawal may result in uniform growth of follicles and an optimum stage of follicle development at sponge removal, due to the importance of LH in the growth and development of the ovulatory follicle(s) (Ginther *et al.*, 2001). Therefore, within a

previous study (Evans *et al.*, 2004) we exposed ewes to rams during the last three days of a progestagen synchronisation programme, undertaken during the breeding season, and examined the impact of this pre-mating ram exposure on LH concentrations (whilst still under the influence of the progestagen) and the timing of the LH surge, oestrus and ovulation (post sponge withdrawal). This previous study (Evans *et al.*, 2004) identified a significant increase in LH concentrations in response to the pre-mating ram exposure and the ram-exposed ewes had a more rapid onset of oestrus, shorter oestrous period and an earlier LH surge and ovulation compared to control ewes. However the number of ram-exposed ewes mated at and conceiving to the synchronised service was lower than that of control ewes. The ram-exposed ewes that did conceive had comparable litter size to control ewes (Evans *et al.*, 2004). Therefore it was suggested that the LH response induced by the pre-mating ram exposure had as hypothesised accelerated follicle development but that this had resulted in some of the ewes reaching the end of their fertile oestrous period when the rams were introduced for mating at 48 hours post sponge removal (Evans *et al.*, 2004). The reduction in overall reproductive performance may also have occurred due to a negative effect of the pre-mating ram exposure on the functional competency of the oocyte, embryo or corpus luteum (Evans *et al.*, 2004).

The aim of this study were to investigate further the negative effects of a pre-mating ram exposure (during the last 3 days of an artificial synchronisation protocol) upon fertility. Specifically, my aim was to determine whether ram introduction at 24 hours post sponge withdrawal eliminates the previously observed depression in conception rates within ram-exposed ewes.

9.3 MATERIALS AND METHODS

9.3.1 ANIMALS AND EXPERIMENTAL PROCEDURES

During October, 133 multiparous mule ewes (Swaledale x Bluefaced/Border Leicester) (which had been previously isolated from ram contact) were treated for 14 days with an intravaginal progestagen pessary (60mg medroxyprogesterone acetate; Veramix sponge, Upjohn, UK) inserted on Day 0 of the experiment. Ewes were maintained on pasture at Cockle Park Research Farm, Newcastle upon Tyne (55°13'N) and were allocated to ram-exposed (SR; n=66) and control groups (SC; n=67), balanced on age and parity. SR ewes commenced their pre-mating ram exposure on Day 11 and rams

(n=14) remained with the ewes for the duration of the progestagen treatment. Control ewes remained isolated from the rams during the experimental period prior to mating. Rams were removed from the SR ewes at sponge withdrawal on the fourteenth day of the experiment.

Twenty four hours after sponge withdrawal, raddled rams were introduced for mating (n=7 per group of 66 or 67 ewes; random mix of SC and SR ewes). Raddle marks were checked at 4-hour intervals from 4 to 72 hours post ram introduction to detect the oestrus. Rams were removed at 72 hours after ram introduction, raddle colour was changed and rams were reinstated with ewes 14 days later to observe any ewes not conceiving to the first service. The ewes were maintained subject to conventional farm practice until lambing when the number of lambs and date of lambing were recorded.

9.3.2 DATA ANALYSIS

Data sets were analysed using the ANOVA procedures of SAS (SAS version 6.12, Cary, North Carolina, USA) or chi square analysis. Data are presented as frequency (%) or the mean ± sem as appropriate.

9.4 RESULTS

There was no difference between SR and SC groups in the number of ewes mated to first service (Table 9.1). Furthermore there was no difference in conception rates to the first service or in the number of ewes first mated at the second service (Table 9.1). Within ewes lambing to first service, more SR ewes yielded single lambs ($P<0.01$; Table 9.1) and this differential in lambs born per ewe resulted in a lower litter size within SR ewes ($P<0.01$). There was no difference between SR and SC groups in the number of ewes lambing to subsequent services or those that were barren, culled or died during the experiment.

Table 9.1. The effect of exposure of ewes to rams during the last three days of progestagen treatment on time of mating, conception rates to first service and litter size ($ P<0.1, * P<0.05,** P<0.01, *** P<0.001)

	Control	+ Ram	P
Number of ewes synchronised	67	66	
Number of ewes mated at 1st service at:			
0-24h from ram introduction (%)	44 (66)	34 (52)	$
24-48h from ram introduction (%)	21 (31)	29 (44)	
48-72 h from ram introduction (%)	0 (0)	1 (2)	
Total for 1st service (%)	65 (97)	64 (97)	
Ewes first mated at second service (%)	2 (3)	2 (3)	
Ewes lambing mated at 1st service at			
0-24h from ram introduction (%)	31 (70)	27 (79)	
24-48h from ram introduction (%)	17 (81)	21 (72)	
48-72 h from ram introduction (%)	0 (-)	1 (-)	
Total to 1st service (%)	48 (74)	49 (77)	
Ewes lambing to 1st service having			
1 lamb (%)	7 (15)	19 (39)	**
2 lambs (%)	30 (61)	23 (47)	
> 2 lambs (%)	11 (23)	7 (14)	
Mean (± sem) number of lambs per ewe (1st service)	2.10 ± 0.10	1.76 ± 0.10	**
Ewes lambing to subsequent services	14 (21)	14 (21)	
Ewes that were barren, aborted, died or were culled	5 (7)	3 (3)	

9.5 DISCUSSION

Within a previous study at the University of Newcastle, Evans *et al.*, (2004) observed a depression in conception rates when ewes underwent a pre-mating ram exposure whilst under the influence of an artificial progestagen but observed no effect on litter size. Evans *et al.*, (2004) had hypothesised that the depression in conception rates was due to the ewes being at the end of their fertile oestrous period when ram introduction for mating was delayed until 48 hours post sponge withdrawal. This hypothesis was based on an observed increase in LH concentrations when ewes were exposed to rams at a comparable stage of the synchronisation protocol that was associated with an advanced onset of oestrus, LH surge and ovulation (Evans *et al.*, 2004). Therefore by not introducing ram exposed to rams until 48 hours post sponge removal (Evans *et al.*, 2004), the oocyte may have been placed at a greater risk of tubal ageing prior to the first opportunity for fertilisation. This theory is supported by the observed deterioration in embryo quality in ewes not introduced to rams until 48 hours post sponge withdrawal (Hawken *et al.*, 2005). Within the current study there was no difference between the numbers of control and ram-exposed ewes mated at or conceiving to the first service which suggests an improved synchrony between mating and ovulation when rams were introduced at 24 hours post sponge withdrawal. Delayed insemination of ewes post sponge removal significantly reduces the number of embryos capable of developing into functional offspring (McEvoy *et al.*, 1995). Therefore, I propose that the absence of a depression in conception rates in this study indicates optimal coordination of the time of mating and ovulation in both the control and ram exposed ewes.

The depression in litter size in ram-exposed ewes observed in this study was not observed by Evans *et al.*, (2004) thus suggesting an effect of the ram introduction itself at 24 hours post sponge withdrawal that in conjunction with the pre-mating ram exposure negatively affected litter size. Lucidi *et al.*, (2001) identified a significant increase in LH concentrations and an advanced onset of the LH surge and ovulation within ewes introduced to rams at 24 hours post sponge withdrawal compared to those isolated from rams post sponge withdrawal. This evidence suggests that additional gonadotrophin production stimulated by ram introduction post sponge withdrawal may have antagonised the effect of the pre-mating ram exposure on follicular development. The time available for folliculogenesis is critical determinant of

ovulation rate as ovulatory follicles are those with sufficiently developed LH receptors within the granulosa and thecal cells at the time of the pre-ovulatory LH surge (Webb and England, 1982; Scaramuzzi *et al.*, 1993). Ewes exposed to rams at a comparable stage of the progestagen synchronisation treatment demonstrated an advanced timing of the LH surge (Evans *et al.*, 2004) comparable to that seen in super-stimulation procedures in cattle. In these studies (D'Occhio *et al.*, 1999) the dominant and more structurally advanced follicles drive the LH surge leaving insufficient time for complete development of all other potential ovulatory follicles (Webb and England, 1982). This effectively reduces the pool of follicles developmentally capable of responding to the LH surge and ovulating, thus depressing the ovulation rate (D'Occhio *et al.*, 1999). Within the ewe, several studies have identified the development of a persistent dominant follicle when ewes are exposed to sub-luteal levels of progesterone that permits elevated levels of LH whilst ewes are still under the influence of the artificial progestagen (Flynn *et al.*, 2000; Evans *et al.*, 2001). This elevation in LH is similar in both timing and magnitude to that observed by Evans *et al.*, (2004) in ewes exposed to rams towards the end of a synchronisation protocol. Therefore, I propose that the ram-induced increase in LH towards the end of the progestagen treatment may cause development of more structurally advanced pre-ovulatory follicles at sponge removal. The size and status of these follicles may have prevented selection of additional ovulatory follicles due to the elevation of oestradiol and inhibin associated with large follicles (Baird and Campbell, 1998). Therefore, I propose that the presence of a large preovulatory follicle at sponge withdrawal that may have developed as a consequence of a ram induced elevation in LH (Evans *et al.*, 2004) prevented selection of further ovulatory follicles that coupled with advanced stimulation of the LH surge resulted in an overall depression of ovulation rate and litter size.

The negative effects of excessive levels of LH during folliculogenesis on embryo quality are reported within synchronisation protocols in cattle (Revah and Butler, 1996; Mihm *et al.*, 1999) and in polycystic ovary syndrome in women (Homburg, 1998; Filcori, 1999). The detrimental effects of prolonged exposure of follicles elevated LH are mediated by premature disruption of granulosa cell communication which causes early resumption of meiosis, abnormal oocyte maturation and ovulation of an aged and inferior oocyte (Filcori, 1999; Mihm *et al.*, 1999) leading to embryo

loss and reproductive wastage. However it is unlikely that the ram induced increase in LH concentrations had any negative effect on oocyte or embryo quality due to the failure to detect any such detrimental effects in eCG treated ram-exposed ewes (Hawken *et al.,* 2005). These observations are also in agreement with Evans *et al,* (2004) where neither oocyte nor embryo quality were affected by prolonged exposure to elevated LH induced by sub-luteal levels of progesterone during the synchronisation protocol.

In summary, the exposure of ewes to rams towards the end of a synchronisation protocol combined with earlier ram introduction (24 hours versus 48 hours post sponge removal) for mating appeared to improve the synchrony between time of mating and ovulation resulting in no difference in conception rates to the synchronised service. I hypothesise however that the depression in litter size observed in this study that was not seen in a previous study when rams were introduced for mating at 48 hours post sponge removal (Evans *et al.,* 2004) was due to the ram introduction at 24 hours itself inducing an elevation in levels of LH. We suggest that this further antagonised the effect of the ram induced elevation in LH prior to sponge withdrawal resulting in a large and dominant pre-ovulatory follicle that stimulated the LH surge at a point where only a limited number of follicles were developmentally capable of responding to the endocrine signal, thus reducing ovulation rate and consequently the number of lambs born per ewe.

10. GENERAL DISCUSSION AND CONCLUSION

The timing of the ram exposures during the transition into the breeding season has been shown in this thesis to be an effective method of stimulating a synchronous onset of cyclic activity in the seasonal mule ewe. I will discuss the reasons for this and the parameters affecting the reliability and predictability of this response. The repeated exposure of ewes to rams after the onset of cyclicity appears to have had some residual affect on the distribution of oestrus at mating. Therefore the endocrine response to ram introduction of cyclic ewes will be discussed in an attempt to fathom the mechanisms and effect of a ram induced increase of LH in cyclic ewes. Finally the ever-complicated maiden ewe and the role of prior experience with the ram will be discussed relative to the observations within this thesis and previous work in this area.

10.1 RAM INTRODUCTION TO EWES DURING LATE ANOESTRUS AND THE TRANSITION INTO THE BREEDING SEASON.

Martin *et al.*, (1986) criticised the use of anoestrus to describe the physiological state of ewes during the non-breeding season due to the requirement of ram presence to detect oestrus and the subsidiary effects of the ram on the physiological state of the ewe. Similarly in this study the terminology for the timing of ram exposures during anoestrus and the transition into the breeding season have similar classification problems. This is due specifically to the effect of socio-sexual cues from rams, con-specifics and also stress on the onset of cyclic activity.

The factors affecting the magnitude and timing of the LH response to ram introduction and interactions between them are shown in Figure 10.1 relative to the onset of cyclic activity in the ewe.

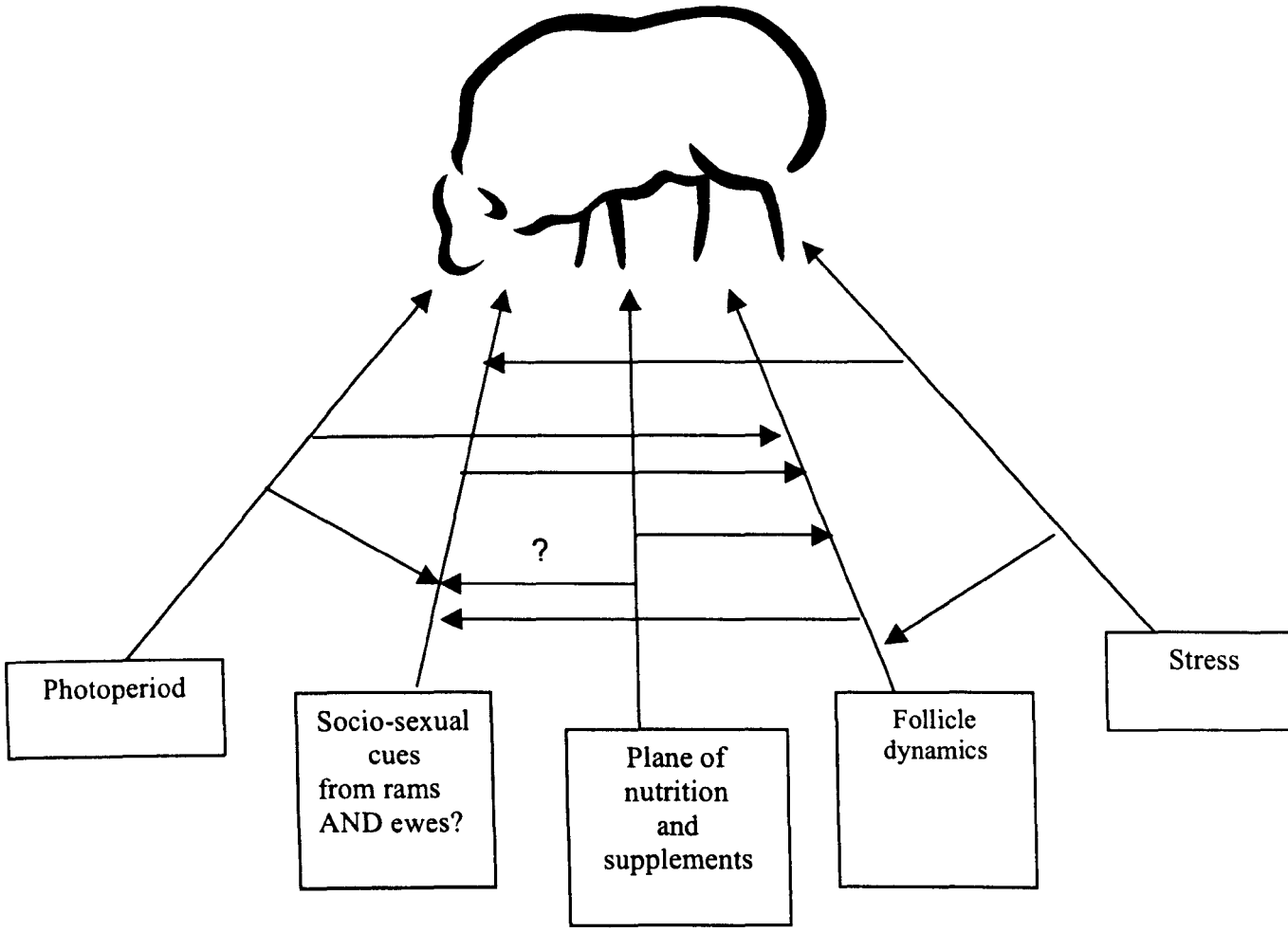

Figure 10.1 Schematic representation of the factors affecting the timing of the onset of cylic activity in the ewe. Vertical arrows indicate a direct effect on the onset of cyclicity in the ewe. Horizontal arrows indicate an interaction between two factors that affects the stimulus value or response of the anovular ewe to that stimulus. A question mark indicates a possible but as yet undetermined interaction between two factors.

10.1.1 THE ONSET OF CYCLIC ACTIVITY

Chapters 5 and 7 illustrated that ram introduction to mule ewes during the transition between anoestrus and the breeding season stimulates an increase in LH pulse frequency and mean and basal LH concentrations. Therefore the introduction of rams to anovular ewes during early September in Chapters 3 and 4 is likely to have stimulated a similar endocrine response. However the proportion of ewes responding to the rams, ovulating and subsequently maintaining a normal pattern of cyclic activity is dependent on the type and duration of ram exposure.

The evidence within Chapter 4 indicates that continued ram presence during the transition into the breeding season is more effective in stimulating the onset of cyclic activity than short-term 24-hour exposure to the ram. However based on the observations in short term ram exposed ewes in Chapters 3 and 4, I propose an association between changing photoperiod and the potency of the 24-hour ram exposure. At the time of the first ram exposure period, ewes exposed to the ram stimulus for only 24 hours that ovulated in response to the ram effect had to be at a specific physiological state relative to follicle dynamics and seasonality to respond to the ram with an LH surge and ovulation. Any ewes not at this optimal state will not ovulate or ovulate but have a short oestrous cycle and in the absence of continuous ram presence return to an anoestrous endocrine state (Pearce and Oldham, 1984). The timing of the second short term ram exposure period closer to the proximity of the natural breeding season but at a time when a high proportion of ewes are still anovular is likely to be a more successful stimulant. This theory is based on previous studies that show a greater ovulatory response to the ram effect closer to the natural breeding season (Oldham and Cognie, 1980; Rosa and Bryant, 2002). A proportion of ewes are again likely to respond with a short oestrous cycle (Martin *et al.* 1986) or a delayed ovulation (Ungerfeld *et al.*, 2002). However I propose that the declining photoperiod will support the maintenance of normal cyclicity. This theory is based on the direct relationship between date of ram joining relative to the natural breeding season and the proportion of ram exposed ewes returning to anoestrus (Oldham and Cognie, 1980). In contrast, ewes maintained in continuous ram presence (Chapter 4) from early September had an earlier onset of cyclic activity and proportionately more ewes having three oestrous cycles prior to mating. This would indicate that in continued ram presence, the physiological state and follicular dynamics of the ewe are less

important in the success of initiation of cyclic activity by the ram and more critical in determining the subsequent distribution of oestrus.

10.1.2. THE LH RESPONSE TO RAM INTRODUCTION

What is important in stimulating the onset of cyclic activity?

I have outlined above the success of the ram in stimulating the onset of reproductive activity in anovular mule ewes. However the LH profiles from ewes in Chapters 5 and 7 show inconsistencies in the LH responses of ewes that are detected or not detected with an LH surge or luteal function within 6 days of ram introduction. Therefore the question is raised as to what characteristics of the ram induced LH response are the most critical to the stimulation of an LH surge and ovulation?

Martin *et al.* (1986) identified LH pulse frequency as the primary determinant of stimulation of ovulation by the ram effect. However within Chapters 5 and 7, LH pulse frequency after ram introduction was not the *sole* determinant of the detection of an LH surge or of luteal activity within 6 days of ram introduction. This in agreement with the findings of Oldham and Pearce (1983) who found that the increase in LH pulse frequency during the first 4 hours of ram introduction was not an accurate indication of whether ewes went on to ovulate or not within 72 hours of ram introduction. Martin *et al.*, (1986) postulated that this might be due to a stress-suppressed response by the process of blood sampling or a gradual but critical response that was not evident within the first few hours of ram introduction.

In Chapter 7, ram naïve ewes had numerically higher basal concentrations of LH in addition to greater LH pulse frequency after ram introduction. These same ewes had an earlier LH surge relative to the time ram introduction and a greater proportion of ewes (38%) were detected with the onset of the LH surge prior to the sampling period (18 hours after ram introduction). Within the model for follicular development proposed by Scaramuzzi *et al.*, (1993) the transition from gonadotrophin-responsive follicles to gonadotrophin–dependent follicles is dependent on increased concentrations LH but it is not critical that it is pulsatile. Therefore I propose that the association between a parallel increase in LH pulse frequency *and* basal LH affects the *timing* (rather than the occurrence) of the LH surge by a synergistic effect on follicle development.

The selection of the sampling period between 18 and 44 hours after ram introduction was based on the review by Martin *et al.*, (1986) where the reported mean timing of the LH surge was between 20 ± 3 and 40 ± 2 hours after ram introduction. However in both Chapters 5 and 7 there were a number of ewes with an onset of cyclic activity within 5 days of ram introduction that were not detected with an LH surge within this time period. This may be at least in part a factor of the stage of follicle development within the follicular wave at the time of ram introduction. Ungerfeld *et al.*, (2002) found that within a sample of anoestrous ewes, 27% did not ovulate in response to the ram effect until Day 6 after ram introduction. Within these ewes the largest follicle present on the day of ram introduction had emerged 3 to 5 days earlier and was in the static or regressing phase when rams were introduced. Therefore the proportion of ewes that showed an acute response to ram introduction but that subsequently were not detected with an LH surge in the sampling period may have been at an inappropriate stage of the follicular wave respond instantly to ram introduction.

The period after ram introduction is typically the main focus of investigations into the ram effect. However within Chapter 7, I observed a significant association between LH concentrations before ram introduction and whether ewes were detected with an LH surge after ram introduction. The depth of anoestrus within more seasonal breeds of ewe is a reflection of LH pulse frequency during the anoestrous period driven by the sensitivity of ewes to the negative effects of oestradiol (Thomas *et al.*, 1984). The ram naïve ewes in Chapter 7 had significantly greater LH pulse frequency than ram experienced ewes both before and after ram introduction and 90% of ewes were detected with an LH surge. This relationship between LH concentrations prior to ram introduction and the magnitude and ovulatory success of the ram-induced response may provide a potential new area of investigation for enhancing the ovulatory response through modification of the endocrine milieu prior to ram introduction.

Therefore I conclude that the characteristics of the LH response after ram introduction can be a good indicator of the relative timing of the LH surge however they are not necessarily a good indication of whether ewes ovulate or not in response to ram introduction. I propose that the pre-ram introduction endocrine milieu, stage of follicle development and the maintenance of an LH response to ram introduction are

the vital aspects in whether a ewe is stimulated to ovulate in response to ram introduction.

10.1.3 THE INFLUENCE OF STRESS

Acute stress, such as transport, can stimulate the onset of cyclic activity in a number of species (pigs: Hughes, 1982; sheep: Braden and Moule, 1964). Within Chapter 3, the ewes maintained in isolation from the ram stimulus (control ewes) that were blood sampled twice weekly for the duration of the pre-mating period had a markedly more compact distribution of mating than control ewes that were maintained undisturbed at pasture. The progesterone data from the blood sampled ewes showed that this compact distribution at mating originated from the onset of the breeding season. Furthermore within the anoestrous serial bleed trials (Chapters 5 and 7) a proportion (5-20%) of anovular ewes had the onset of the first oestrous cycle of the breeding season during the blood sampling period prior to ram introduction. Van Lier *et al.*, (1998) identified a direct relationship between handling of sheep and an increase in cortisol. The trauma associated with the simple process of handling is further highlighted by the work of Khalid *et al.*, (1998) where handling was associated with the greatest stress response, irrespective of whether ewes were subsequently inseminated by laparoscopic or cervical artificial insemination.

The mechanism through which stress stimulates this onset of cyclic activity remains unclear and there is much contradiction in the literature as to positive or negative effects of stress on reproductive function in sheep (Review, Tilbrook *et al.*, 2002). It has been proposed that there is a rebound of GnRH release after habituation to the stressor and that this rebound in gonadotrophin release is sufficient to stimulate ovulation in an anoestrous animal (Tilbrook *et al.*, 2002). This is of particular relevance during the transition into the breeding season where elevation in LH over an equivalent duration of the follicular period can be sufficient to induce ovulation and the first oestrous cycle of the breeding season (Karsch, 1980). Furthermore sporadic increases in progesterone during the transition into the breeding season play a critical role in synchronising follicular dynamics and typically occur within 1 week of the onset of the first cycle of the breeding season. Van Lier *et al.*, (1998) identified a parallel increase in extragonadal progesterone in response to infusion of cortisol and a direct association between the process of handling and extragonadal progesterone.

Therefore during this transitional period, sporadic rises in progesterone induced by stress may have affected the onset of cyclicity.

This therefore raises the question as to the comparative benefit of the use of vasectomised rams for the ram exposure periods over fence line ram exposure observed within Chapter 3. Anoestrous ewes respond to the ram effect with an optimal endocrine and ovulatory response when they are exposed to the full complement of socio-sexual cues associated with the ram (Pearce and Oldham, 1988). The compaction in breeding was comparatively greater in the ewes exposed repeatedly to the vasectomised rams thus supporting this theory, however as outlined above there was also a marked compaction in the distribution of the control ewes. The question therefore arises as to the contribution of stress to the compacted distribution of mating within the vasectomised ram exposed ewes. To determine if this were the case, a further experiment would have to be conducted that directly compared the vasectomised and fence-line ram exposures in the absence of blood sampling.

10.1.4 THE EFFECTS OF NUTRITION

Nutrition has a more important role in determining the 'seasonality' in breeds of sheep native to less temperate climates (Karsch *et al.,* 1984) and is a critical determinant in the ovulatory response of goats and tropical breeds of sheep to the male effect. However the role of nutrition in modulating the responses of ewes to the ram effect is more questionable (Knight, 1983). Nonetheless the capacity for nutrition to affect the endocrine responses of bucks to the female effect (Walkden-Brown *et al.,* 1994) and evidence in rams of a direct modulation of LH pulse frequency (Boukhliq and Martin, 1997) postulates that there may be a more primary role of nutrition than that previously perceived. Ewes in Chapter 7 had enhanced aspects of the LH response both before and after ram introduction than maiden ewes in Chapter 5 and though no direct comparison can be made due to year differences the most marked difference between the two years (aside from prior ram experience) was nutritional supplementation. An effect of nutrition may be dependent on the physiological state of the ewe at the time of supplementation. However the capacity for nutritional manipulation of the endocrine milieu prior to ram introduction may present a mechanism for overriding the 'depth of anoestrus' of seasonal breeds of sheep.

10.2 THE EFFECT OF RAM INTRODUCTION TO CYCLIC EWES

Chapter 6 identified that introduction of a ram during the breeding season to randomly cyclic ewes stimulates an LH response irrespective of the stage of the oestrous cycle at the time of ram introduction. This is the first demonstration of the ability of ram exposure to elicit an endocrine response in randomly cyclic ewes. However the limited numbers of ewes in each stage of the oestrous cycle restricted statistical analysis to ewes in the early and late luteal phase. The magnitude and characteristics of the response of ewes in the late luteal phase are comparable to those observed after ovulation in pregnant ewes by Al-Gubory (1998). During the luteal phase pulsatile LH is suppressed by the synergistic action of progesterone and oestradiol on GnRH release (Karsch *et al.*, 1977). Pearce and Oldham (1983) proposed that an increase in LH pulse frequency in ovariectomised ewes (implanted with progesterone and oestradiol) was mediated though a reversal of the negative effects of progesterone and oestradiol on LH release. However Pearce and Oldham (1983) also suggested a steroid independent mechanism based on the increase in basal LH in ram exposed ovariectomised ewes not implanted with steroids. This basal increase is similar to that observed in untreated ovariectomised anoestrous ewes (Martin *et al.* 1983c) and may suggest a dual mechanism in the stimulation of LH release by the ram.

Within Chapter 3, I observed a greater compaction of mating than that observed at the onset of the breeding season and hypothesised that this developed during the pre-mating period through an effect of the ram on cycle length. The capacity for the cyclic mule ewe to respond to ram introduction with an increase LH supports several of the mechanisms outlined in Chapter 3 that I proposed may be altering the distribution of oestrous cycles during the breeding season. Furthermore within Chapter 4, the digression in the median date of oestrus in ewes maintained in continuous ram presence and introduced to novel rams at the same 17-day interval further supports the capacity for periodic ram introduction to alter the distribution of mating within cyclic ewes. This also raises the issue of novelty; as to whether the ewes maintained in continuous ram presence had an LH response when novel rams were introduced and the possible effect of this on follicle development and characteristics of the oestrous cycle.

Figure 10.2 summarises the proposed possible mechanisms of the effects of ram introduction on ewes at different stages of the oestrous cycle. The possibility of a luteolytic role of LH at the end of the luteal phase is of particular interest in this mechanism based on the decline in progesterone over the serial bleed period at a rate greater than that associated with luteolysis and the occurrence of an LH surge. This area requires further investigation to offer anything other than speculative conclusions. However within Chapter 6 the ewes that had a rapid decline in progesterone after ram introduction and were detected with an LH surge were also those that responded with a defined and large increase in LH pulse frequency to ram introduction.

The presence of only one ewe in the follicular phase restricts any meaningful discussion of the effects of the ram at this time. However evidence of an advanced LH surge relative to the timing of oestrus in cylic ewes identified by Lindsay *et al.*, (1975) in addition to the observed increase in basal and mean LH concentrations in Chapter 6 infers that ram introduction at this time has the capacity to affect the time of ovulation. This is supported in the observation in ram synchronised ewes in Chapter 8 where ewes marked during the oestrous cycle prior to artificial insemination had significantly lower conception rates compared to ewes first marked by the vasectomised rams immediately prior to mating. I proposed that this was due to asynchrony between ovulation and mating due to the repeated exposure of oestrous ewes to the ram resulting in a cumulative shift in the timing of ovulation relative to the first ram exposure period. This theory is supported by the large proportion of repeated fence line ram exposed ewes in Chapter 3 that did not conceive to the first service when mated during the first day after entire ram introduction; 17 days after the last ram exposure period. Furthermore the permanent ram exposed ewes in Chapter 4, had a persistent significant decline in the number of days from raddle colour change to marking by the vasectomised rams. This decline in the absence of additional or novel stimuli infers a possible persistent effect of the ram during the follicular phase, even in ewes maintained in continuous ram presence.

The capacity of the ewe to respond with an endocrine response to ram introduction during the breeding season opens a realm of applications for the ram effect that have previously been left relatively unexplored. However the potency for the ram to affect

the distribution of oestrus either by affecting the onset of the breeding season or through an effect on cyclic ewes may have residual consequences on subsequent litter size when entire rams are introduced for mating. Studies in anoestrous ewes have indicated a positive effect of ram introduction on ovulation rate (Cognie *et al.*, 1980; Pearce and Oldham, 1984). However these studies have recurrently been without an adequate control (Martin *et al.*, 1986) and in contrast to the studies in this thesis, have been predominantly in the anoestrous Merino ewe. The mule ewe is markedly more prolific than the Merino ewe with a comparative lambing percentage of 158% (Cognie *et al.*, 1980) compared to 200% (Control ewes; Chapter 3).

Based on the observations in Chapters 3 and 4, I propose not necessarily a direct effect of the ram induced increase in LH on litter size but an effect mediated by the ram driven distribution of oestrus relative to the time of entire ram introduction. Within Chapter 3, repeated ram exposed ewes tended to have a lower litter size than ewes isolated from rams prior to mating. Within Chapter 4, repeated ram exposed had a significantly lower litter size than single ram exposed ewes. I outlined two mechanisms through which the repeated ram exposure may have resulted in the increased frequency of single and reduced frequency of multiple births (Chapter 4). Both of these mechanisms related specifically to the distribution of mating when entire rams were introduced during the breeding season driven by an effect of the ram on the timing of the LH surge relative to luteolysis. The capacity for elevated LH towards the end of the luteal phase to affect litter size by a depression in ovulation rate is evident in Hawken *et al.*, (2005) and Chapter 9. Within Chapter 9, I hypothesised that the reduction in litter size in ram exposed was driven by an effect of the ram-induced increase in LH on follicle development prior to progestagen withdrawal.

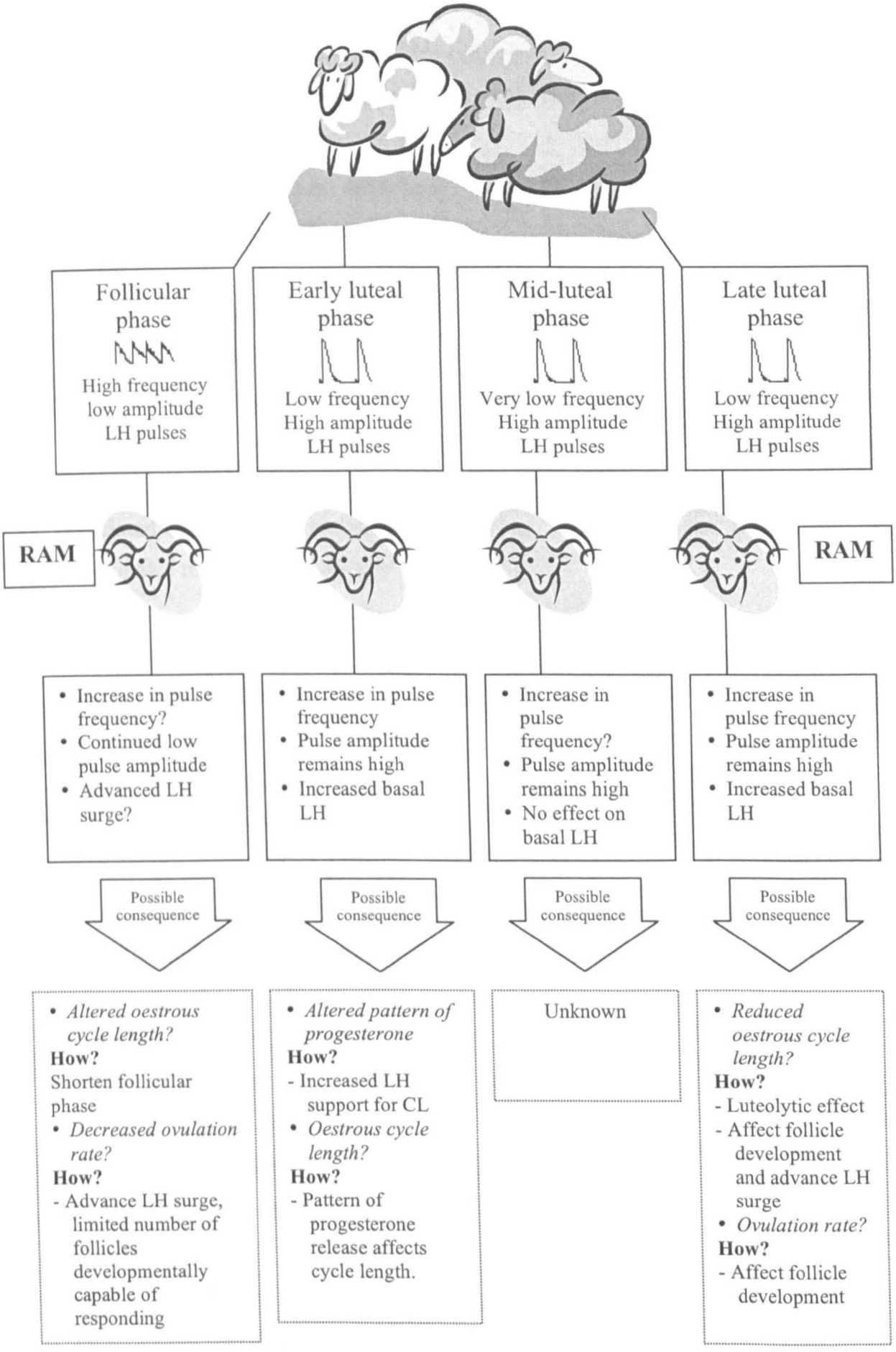

Figure 10.2. Summary of the effects and possible consequences of ram introduction to cyclic ewes

10.3 THE ROLE OF PRIOR EXPERIENCE WITH THE RAM ON THE ENDOCRINE AND BEHAVIOURAL RESPONSES OF THE EWE TO SUBSEQUENT RAM INTRODUCTION.

Chapters 5 and 6 showed no difference in any parameters of the LH response to ram introduction in ewes with or without prior experience with the ram during the anoestrous period. This is in contrast to my initial hypothesis that prior experience with the ram would enhance the endocrine responses of the maiden ewe to ram introduction. However prior experience with the ram during anoestrus did modulate the *behavioural* responses of maiden ewes. Ram experienced maiden ewes showed more ram seeking behaviour, less avoidance behaviour of the ram and spent more time in close proximity of the ram. A summary of the roles of the various factors associated with the neuroendocrine and behavioural responses of the sexually naïve maiden ewe to the ram is outlined in Figure 10.3.

Behavioural responses to introduction of a potential mate typically require a degree of learning in both males and female (Review, Gelez and Fabre-Nys, 2004). This is true of the ram (Kridli and Said, 1999) and the importance of sexual experience in the development of appropriate oestrous behaviour may be the primary origin of the reduced level of fertility observed in maiden ewes. This theory is based on the absence of a pattern of neurotransmitter release in the medial basal hypothalamus that is critical to receptivity and proceptivity of the ewe to the ram (Gelez *et al.*, 2004a). However within Chapter 5, the maiden ewes were not mounted by the vasectomised rams during the pre-conditioning ram exposure period, which is thought to be critical in the development of the pattern of neurotransmitter release outline above. Therefore modification of the behaviour of maiden ewes by mere exposure to the ram indicates a direct effect of the socio-sexual cues on the subsequent behavioural and social ram to ewe interactions. I propose that this may be due to a reduced fear of the ram or identification of the ram as a potential mate.

Within rodents, endocrine responses to the opposite sex are elicited independent of prior sexual experience (Vandenburgh, 1974). The absence of an effect of prior experience with the ram on the endocrine responses of maiden ewes observed in this thesis is in agreement with those of Gelez *et al.*, (2004c) who found no difference in the endocrine responses of sexually naïve and experienced maiden ewes to ram

introduction. The capacity for the ram to advance the onset of puberty (Al-Mauly *et al.*, 1991) and the significant increase in parameters of the LH response in response to ram introduction in all ram naïve ewes in this thesis indicates an innate ability of the maiden ewe to respond to ram introduction. This is in agreement with the female effect in rams, where the endocrine response to the introduction of oestrous ewes is not dependent on prior sexual experience (Gonzalez *et al.* 1991). However it is of interest that this endocrine response is enhanced by prior sexual experience (Gonzalez *et al.*, 1991; Borg *et al.* 1992). Within Chapter 5 there was a tendency for maiden ewes with prior experience with the rams during anoestrus to have an enhanced increase in LH pulse frequency and a greater frequency of normal length oestrous cycles after ram introduction. Though these observations were neither significant nor represented in the ram-experienced ewes in Chapters 6 or 7, this does highlight the relative uncertainty as to the role of prior experience in the endocrine responses to ram introduction. As it is not an absolute effect of occurrence or non-occurrence of the LH response in addition to individual within breed variation it is difficult to ascertain whether the trends observed in Chapter 5 are important are not.

From Chapters 5, 6 and 7, I have determined that the endocrine response to the ram is not dependent on prior experience with the ram. However it would appear that stimulation of the ewe using only the scent of the ram is a learned response. Gelez *et al.*, (2004c) found that ram experienced maiden ewes responded to exposure to rams fleece with a significant increase in LH pulse frequency compared to ram naïve maiden ewes that did not respond with an increase in LH pulse frequency. The capacity for olfactory learning in the ewe was demonstrated relative to the endocrine response to the ram by Gelez *et al.*, (2004c) by preconditioning sexually naïve maiden ewes to males or females scented with lavender and males not scented with lavender. On subsequent exposure to a lavender scented female fleece, ewes previously maintained with lavender scented males showed a characteristic increase in LH pulse frequency that was not evident in ewes maintained with lavender scented females of non-lavender scented males (Gelez *et al.*, 2004c). Therefore I propose that the critical factor associated with learning or experience in the endocrine response to the ram is the capacity for the ewe to respond to any one of the stimuli associated with the ram with a comparable endocrine response to that stimulated by introduction of a ram.

However the ram naïve ewes exposed to rams fleece alone did show a significant increase in basal LH. This may suggest that the steroid independent response to ram introduction evident in ovariectomised ewes during both anoestrus (Martin et al., 1983c) and the breeding season (Pearce and Oldham, 1983) may be mediated by a different mechanism. The accessory olfactory system cannot substitute for the main olfactory system in mediating the olfactory response to ram introduction (Gelez and Fabre-Nys 2004). However Fos activation of both olfactory systems when rams are introduced (Gelez and Fabre-Nys 2004) indicates that it does have a role in the endocrine response. Therefore based on the association between this system and the innate responses to primer pheromones, I propose that the ram induced increase in basal LH may be mediated through the accessory olfactory system.

Within Chapter 7, the significantly greater LH pulse frequency both before and after ram introduction in the ram naïve ewes illustrates a different pattern to that observed within Chapters 5 and 6. The ram naïve ewes repeatedly had (non-significantly) higher pulse frequency before ram introduction however the pulse frequency after ram introduction was parallel to that observed in the ram experienced ewes. The reason for this is unclear and though I proposed a possible role of the proximity of the pre-conditioning ram exposure to the onset of anoestrus, the differences in pulse frequency before ram introduction may be merely due to chance.

An additional complicating factor in the role of prior male experience is related to the management of ewes between birth and puberty. Within Chapters 5, 6 and 7, I have proposed that the ewes are ram naïve, however as they are bought into the research facility, I have no information on their management pre-weaning. In male mice, rearing in mixed sex groups affects the sexual competency of mice however this is typically due to the presence of oestrous females within the group (Woodson 2002). However though male and female lambs are unlikely to be maintained in mixed groups post puberty due to the likelihood of untimely fertilisation, the maintenance of lambs prior to weaning in mixed groups may modify subsequent behavioural and endocrine responses to the ram.

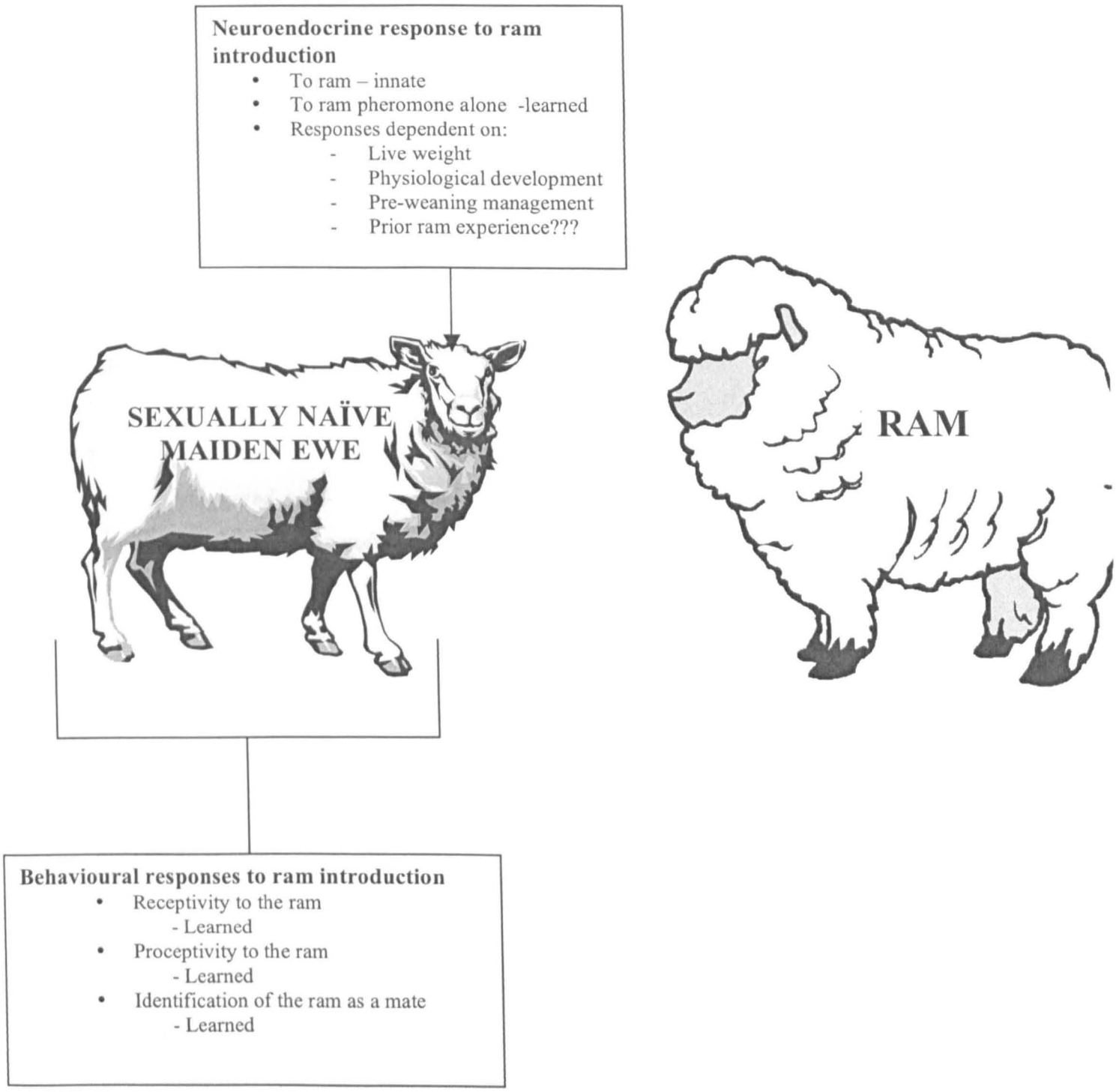

Figure 10.3 Summary of endocrine and behavioural responses of the sexually naïve maiden ewe to the ram

10.5 FUTURE AREAS OF STUDY

The studies within this thesis have highlighted several areas that require further investigation to determine the presence and relevance of certain effects.

The effects of ram introduction during the breeding season require definitive investigation to determine the effects of ram introduction to ewes at different stages of the oestrous cycle. The primary problem that I encountered in monitoring the effects of ram introduction on oestrous cycle length during the breeding season (Chapters 3 and 4) was due to the twice-weekly blood sampling regime. The possible change in cycle length stimulated by the ram is likely to be gradual and easily masked by this relatively low frequency of sample collection. Furthermore in Chapter 6 the unbalanced and small numbers of ewes at each stage of the oestrous cycle restricted the analysis of the effect of ram introduction on LH release in cyclic ewes. I would propose staggered artificial synchronisation of groups of ewes (100 ewes per group) to obtain groups at different stages of the oestrous cycle at sponge removal. After a delay of one oestrous cycle length (to avoid any confounding effects of progestagen synchronisation) entire rams would be introduced and raddle marks recorded daily for two oestrous cycle lengths from the date of entire ram introduction. I would then follow the ewes through to lambing to deduce any effects of the ram on litter size. A subset of each treatment group would be slaughtered on Day 5.5 after mating and the uterine tracts recovered and assessed for the number of corpora lutea and embryo quality as in Hawken *et al.*, (2005). A further subset (10-12 ewes per group) of the each group would be removed prior to ram introduction to the main flock to undergo a frequent blood-sampling regime for 6 hours before and 6 hours after ram introduction. Maintenance of the frequency of the blood sampling at every 12 minutes would be sufficient for ewes in the luteal phase, however based on observations in Chapter 6, this sampling frequency would be insufficient to detect the rapid low amplitude pulses associated with the follicular phase of the oestrous cycle. For ewes in the follicular and late luteal phase, two hourly samples would be taken from the cessation of the frequent sampling period to 72 hours after ram introduction to detect an LH surge. Therefore to detect definitive pulses at this stage of the oestrous cycle, sampling frequency would need to be set at every 2-4 minutes (Martin *et al.*, 1986). I would use ultrasound to monitor follicle emergence and development before and after ram introduction. Ultrasound scanning has been well established to provide an accurate

assessment of follicle development (Ungerfeld *et al.*, 2002; Vinoles *et al.* 2003). The use of ultrasound would provide an indication of the events leading up to ovulation and possible explanations for any group differences observed in the uterine tracts at slaughter.

The issue of prior experience with the ram still requires further investigation to draw definitive conclusions as to any effect of prior ram exposure on the endocrine response to ram introduction. This area is very difficult to conclusively assess due to the interactions with age, live weight, stage of physiological development and seasonality. Within the studies in this thesis, I were restricted by the numbers of ewes due to the logistics of taking frequent blood samples from a large number of ewes. I would propose a series of carefully executed experiments using maiden ewes and primiparous ewes. Primarily I would propose a longer (1 month) ram exposure period during mid-anoestrus and the timing of the breeding season ram exposure period (1 month) earlier in the breeding season to avoid any confounding effects of the transition between the breeding season and anoestrus. The rams used for these exposure periods should be raddled and the frequent blood samples taken from the ewes before and after ram introduction to determine whether they respond to ram introduction with an LH response during this pre-exposure period. When the rams are subsequently introduced during late anoestrus, I propose assessment again of the LH profile before and after ram introduction in combination with monitoring concentrations of oestradiol and follicle development using ultrasound scanning. I propose that this would allow correlation between the specific characteristics of the LH surge and follicle development. The difficulties that I encountered in accurately determining the timing of the LH surge could be overcome by an extended period of two hourly samples from immediately after the cessation of the frequent sampling period up to 72 hours after ram introduction. The inclusion of primiparous ewes would permit conclusions to be drawn between the ram experienced, ram naïve and adult ewes that have been mated, conceived and lambed.

Within Chapter 4, I hypothesised high proportion of ewes initially stimulated by the 24 hour ram exposure period affected the onset and possibly subsequent distribution of oestrus in the remaining anovular ewes. Studies into the female to female effect have focused on the effect of anovular females and though evidence is equivocal,

O'Callaghan *et al.*, (1994) identified a non-significant increase in LH pulse frequency and 2-day advance on the onset of cyclic activity. I propose that there may be a positive association between the stimulus value of oestrous ewes and the proximity of the natural breeding season. Based on the observations in Chapter 4 in the single ram exposed ewes I also propose an critical role of the proportion of ewes in oestrus at any one time on their cumulative effect on anovular ewes. Furthermore based on the capacity for females within other species (McClintock, 1973) to affect the distribution of oestrous cycles, I hypothesise that introduction of a high proportion of ewes at the same stage in the oestrous cycle may affect the cyclic distribution of ewes.

To investigate the above theories, I would propose a flock scale trial composed of two levels of the intensity of oestrous ewe contact (50 and 75%) and the three levels of proximity to the breeding season (early September and late September and mid October). These dates are proposed based on observations in Chapters 3 and 4 of a large proportion of ewes being anovular but markedly more responsive to ram introduction than during early September and most ewes being cyclic by the beginning of October. The oestrous ewes would be artificially synchronised one oestrous cycle prior to introduction again so that it is a natural rather than an artificially induced oestrus that could affect the signals from the female. Progesterone concentrations should be measured daily to determine definitively if there is any effect of oestrous ewes on the onset of cyclic activity and distribution of oestrous cycles once ewes are cycling. Raddle mark data after the introduction of entire rams will support this data by indicating the distribution of oestrus within ewes exposed at different times and to different levels of oestrous ewe contact. However the ram introduction itself may affect the distribution hence the importance of the daily progesterone data and would have to be considered in interpretation of the results. A schematic protocol diagram is shown in Figure 10.4.

There are several further areas that I believe should be further investigated as listed below:

- The endocrine response to novel ram introduction during different stages of the oestrous cycle and in anovular ewes maintained in continued ram presence.
- The effect of different planes of nutrition on the LH pulse frequency before and after ram introduction and the ovulatory response to the ram effect.

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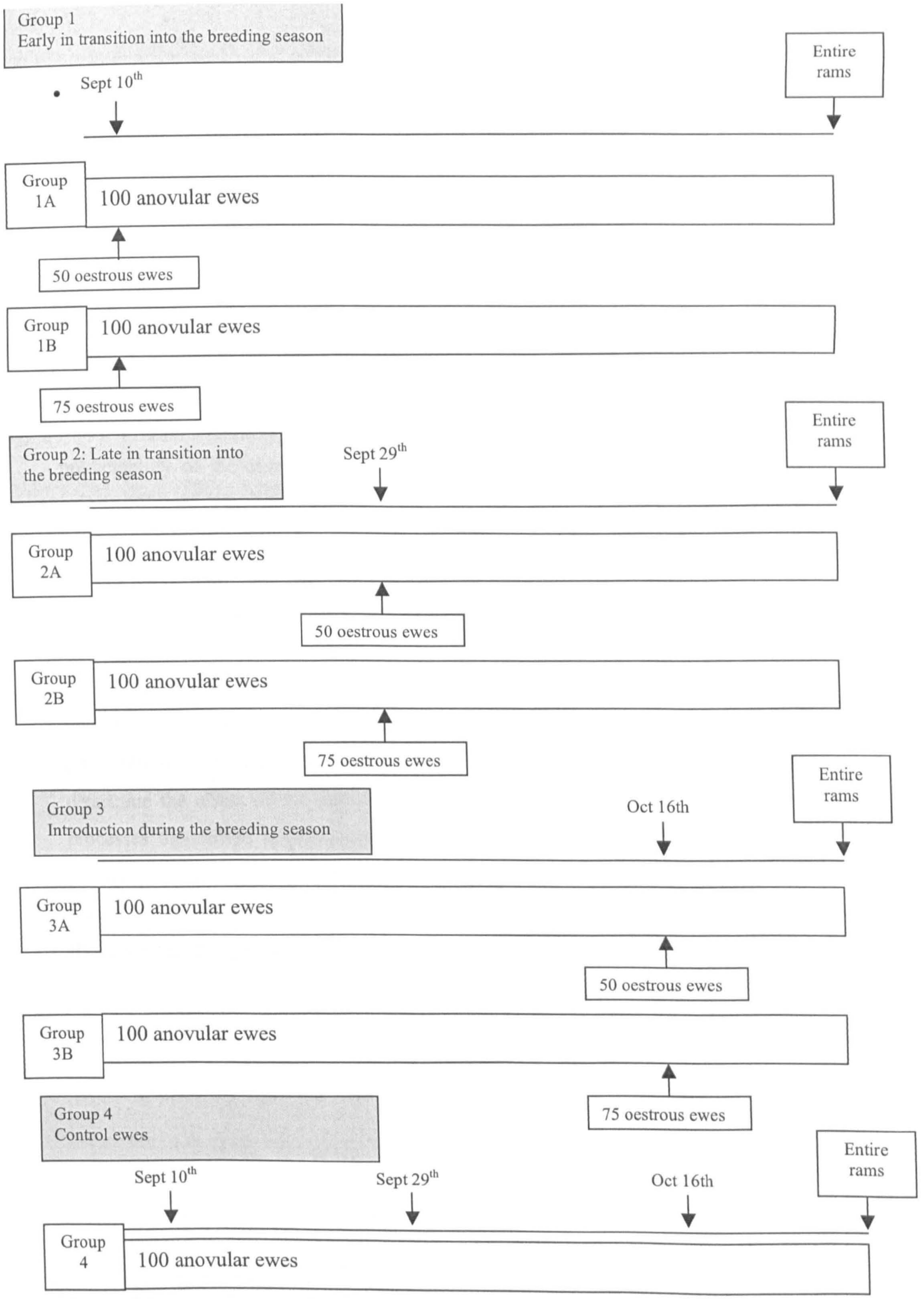

Figure 10.4 Schematic diagram of an investigation into the female-to-female effect in anovular and cyclic ewes

10.6 CONCLUSION

The capacity of the mule ewe to respond to ram introduction with an endocrine response during the transition into and during the breeding season illustrates the potential for manipulation of reproduction using socio-sexual cues in UK sheep production systems. However altering and focusing the distribution of reproductive activity will also amplify any positive or negative effects of an external factor on ewe fertility. This could be applied positively through incorporation of the pre-mating strategies outlined in this thesis with a period of focused feeding. Martin *et al.*, (2004) identified the potential for specific timing of nutritional supplementation during the oestrous cycle in increasing ovulation rate and reducing early embryo loss. The predictability of the distribution of oestrus and flexibility in the timing of mating permitted by these breeding season strategies, make them particularly relevant to this type of nutritional enhancement of fertility. Conversely any negative effect on fertility such as the possible ram induced depression in litter size is also amplified due to the large numbers of ewes all at the same stage of the oestrous cycle.

A difficult aspect in the investigation of the effect of socio-sexual cues on reproduction in sheep is the effect of stress on the parameters measured and observations made. Handling and blood sampling of ewes is critical to accurately determine the effect of the ram on the endocrine state of the ewe. However these processes themselves appear to have the capacity to alter the endocrine state and parameters associated with the onset of cyclic activity and timing of oestrus. Therefore the relevance of the endocrine response observed in blood sampled ewes to that occurring in a parallel group of undisturbed ewes is questionable.

11. REFERENCES

Aba MA, Quiroga MA, Auza N, Forsberg M and Kindahl H (1999). Control of ovarian activity in Llamas (Lama glama) with medroxyprogesterone acetate. *Reproduction in Domestic Animals*; 34:471-476

Acritopoulou S, Haresign W, Foster JP and Lamming GE (1977). Plasma progesterone and LH concentrations in ewes after injection of an analogue of prostaglandin $F_{2\alpha}$. *Journal of Reproduction and Fertility*; 49:337-340

Al-Gubory KH (1998). Effects of the presence of rams during pregnancy on lambing performance in ewes. *Animal Reproduction Science*; 52:205-211

Al-Mauly NZN, Bryant MJ and Cunningham FJ (1991). Effect of introduction of rams on the pulsatile release of luteinising hormone and the onset of reproductive activity in ewe lambs. *Animal Production*; 53:209-214

Al-Shorepy and Notter DR (1996). Genetic variation and covariation for ewe reproduction, lamb growth, and lamb scrotal circumference in a fall-lambing sheep flock. *Journal Of Animal Science*; 74(7); 1490-1498

Alvarez L, Martin GB, Galindo F and Zarco LA (2003). Social dominance in goats affects their response to the male effect. *Applied Animal Behaviour Science*; 84:119-126

Arave CW and Albright JL (1976). Social rank and physiological traits of dairy cows as influenced by changing group membership. *Journal of Dairy Science*; 59:974-981

Baird DT (1978). Pulsatile secretion of LH and ovarian oestradiol during the follicular phase of the sheep estrous cycle. *Biology of Reproduction*; 18: 359-364

Baird DT and McNeilly AS (1981). Gonadotrophic control of follicular development and secretion in the sheep oestrous cycle. *Journal of Reproduction, Fertility and Development Supplement*; 30:119-133

Baird DT and Campbell KC (1998). Follicle selection in sheep with breed differences in ovulation rate. *Molecular and cellular Endocrinology*; 145: 89-95

Bartlewski PM, Beard AP and Rawlings NC (1999). Ovarian function at the onset of the breeding season. *Animal Reproduction Science*; 57:67-88

Bathaei S (1996). Breeding season and oestrous activity of Iranian fat tailed Mehraban ewes and ewe lambs. *Small Ruminant Research*; 22:13-23

Beach FA (1947). A review of physiological and psychological studies of sexual behavior in mammals. *Physiological Reviews*; 27:240-307

Beard AP and Hunter M (1994). Effects of progesterone pretreatment on the oxytocin receptor concentration and the response to oxytocin during the simulated early luteal phase in the ovariectomized ewe *Journal of Reproduction and Fertility*; 102:57-63

Berg KA and Aaamdal J (1991). Artificial insemination with frozen semen in ewes at different time of the breeding season. *Reproduction in Domestic Animals*; 26:27-30

Borg KE, Esbenshade KL, Johnson BH, Lunstra DD and Ford JJ (1992). Effects of sexual experience and mating stimuli on endocrine concentrations in the adult ram. *Hormones and Behavior*; 26:87-109

Boscos CM, Samartzi FC, Dellis S, Rogge A, Stefankis A and Krambovitis E (2002). Use of progestagen–gonadotrophin treatments in estrus synchronisation of sheep. *Theriogenology*; 58 (7): 1261-1272

Boukhliq R and Martin GB (1997). Administration of fatty acids and gonadotrophin secretion in the mature ram. *Animal Reproduction Science*; 49:143-159

Braden AWH and Moule GR (1964). Effects of stress on ovarian morphology and oestrous cycles in ewes. *Australian Journal of Agricultural Research*; 15: 937-949

Brennan PA and Keverne EB (2004). Something in the air? New insights into mammalian pheromones. *Current Biology*; 14 (2) R81-R89

Bright PA, Melise TR and Smythe OE (1998) Artificial insemination of ewes with fresh semen; *Bulletin 4290* Department of Agriculture, Western Australia

Brooks AN, Lamming GE, Lees PD and Haynes NB (1986). Opioid modulation of LH secretion in the ewe. *Journal of Reproduction and Fertility*; 76: 693-708

Bruce HM (1960). A block to pregnancy in the mouse caused by proximity of strange males *Journal Of Reproduction And Fertility*; 1:96-103

Cahill LP, Buckmaster JM, Cumming IA, Parr RA and Williams AH (1974). The effect of the presence of a ram on the time of ovulation in ewes. *Journal of Reproduction and Fertility*; 40:475-477

Cahill LP, Saumande J, Ravault JP, Blanc M, Thimonier J, Mariana JC and Mauleon P (1981). Hormonal and follicular relationships in ewes of high and low ovulation rates. *Journal of Reproduction and Fertility*; 62:141-150

Chemineau P (1983). Effect on oestrus and ovulation of exposing Creole goats to the male at three times of the year. *Journal of Reproduction and Fertility*; 67:65-72

Chemineau P, Levy F and Thimonier J (1986). Effects of anosmia on LH secretion, ovulation and oestrous behaviour induced by males in the anovular Creole goat. *Animal Reproduction Science*; 10(2): 125-132

Chemineau P (1987). Possibilities for using bucks to stimulate ovarian and oestrous cycles in anovulatory goats-a review. *Livestock Production Science*; 17:135-147

Chemineau P, Daveau A, Locatelli A and Maurice F (1993). Ram induced short luteal phases – effects of hysterectomy and cellular composition of the corpus luteum. *Reproduction Nutrition and Development*; 33:252-261

Clarke IJ (1984). Neuroendocrine control of the ovine oestrous cycle in Reproduction in sheep, Editors: Lindsay DR and Pearce DT, Cambridge University Press: 1-6

Clarke IJ, and Cummins JT (1985). GnRH pulse frequency determines LH pulse amplitude by altering the amount of releasable LH in the pituitary glands. *Journal of Reproduction and Fertility*; 73:425-431

Cognie Y, Gayerie F, Oldham CM and Poindron P (1980). Increase ovulation rate at the ram induced ovulation and its commercial application. *Proceedings of the Australian Society for Animal production*; 13:80-82

Cohen-Tannoudji J, Locatelli A and Signoret JP (1986). Non-pheromonal stimulation by the male of LH release in the anoestrous ewe. *Physiology and Behavior*; 36:921-924

Cohen-Tannoudji J and Signoret JP (1987). Effect of short exposure to the ram on the later reactivity of anoestrous ewes to the male effect. *Animal Reproduction Science*; 13(4) 263-268

Cohen-Tannoudji J, Lavenet C, Locatelli A, Tillet Y and Signoret JP (1989). Non-involvement of the accessory olfactory system in the LH response of anoestrous ewes to male odour. *Journal of Reproduction and Fertility*; 86:135-144.

Cohen-Tannoudji J, Einhorn J and Signoret JP (1994). Ram sexual pheromone: First Approach of chemical identification. *Physiology and Behaviour*; 56: 955-961.

Compendium of organic standards May 2004
http://www.defra.gov.uk/farm/organic/legislation-standards/compnedium-May04.pdf

Coquelin A and Bronson FH (1979). Release of luteinising hormone in male mice during exposure to females; habituation to the response. *Science*; 206: 1099-1100

Corke DG (1980) Use of teasing in an AI programme. *Animal Production in Australia*; 13: 81-82

Cumming IA, Buckmaster JM, Blockey MA, Goding JR, Winfield CG and Baxter RW (1973). Constancy of the interval between luteinising hormone release and ovulation in the ewe. *Biology of Reproduction*; 9:24-29

Cushwa WT, Bradford GE, Stabenfeldt GH, Berger YM and Dally MR (1992). Ram influence on ovarian and sexual activity in anoestrous ewes: effects of isolation of ewes before joining and date of ram introduction. *Journal of Animal Science*; 70:1195-1200

Dalton JC, Nadir S, Bame JH, Noftsinger M, Nebel RL and Saacke RG (2001). Effect of time of insemination on the number of accessory sperm, fertilization rate and embryo quality in non-lactating dairy cattle. *Journal of Dairy Science*; 84:2413-2418

Dewsbury DA (1981). Effects of novelty on copulatory behaviour: The Coolidge effect and related phenomena. *Psychological Bulletin*; 89:464-482

D'Occhio MJ, Jillella D and Lindsay BR (1999). Factors that influence follicle recruitment, growth and ovulation during ovarian super stimulation in heifers: Opportunities to increase ovulation rate and embryo recovery by delaying the exposure of follicles to LH. *Theriogenology*; 51: 9-35

Doney JM, Gunn RG, Smith WF and Carr WR (1976). Effects of pre-mating environmental stress, ACTH, cortisone acetate or metyrapone on oestrus and ovulation in sheep. *Journal of Agricultural Science*; 87:127-132

Donovan A, Hanrahan JP, Lally T, Boland MP, Bryne GP, Duffy P, Lonergan P and O'Neill DJ (2001). Artificial insemination using frozen thawed semen. *Teagasc report* January 2001.

Donovan A, Hanrahan JP, Kummen E, Duffy P and Boland MP (2004). Fertility in the ewe following cervical insemination with fresh or frozen thawed semen at a natural or synchronised oestrus. *Animal Reproduction Science*; 84 (3-4); 359-368

Doty RL (1976). Olfaction in mammals. *Mammalian Olfaction, Reproductive Processes and Behaviour*, Academic Press, New York, USA

Dyrmundsson OR and Lees JL (1972). Effect of rams on the onset of reproductive activity in Clun Forest ewe lambs. Journal of Agricultural Science, Cambridge; 79:269-271

Dyrmundsson OR (1973). Puberty and early reproductive performance in sheep. I Ewe lambs. *Animal Breeding Abstracts*; 41(6) 273-289

Edey TN, Kilgour R and Bremner K (1978). Sexual behaviour and reproductive performance of ewe lambs at and after puberty. *Journal of Agricultural Science*; 90: 83-91

Ekkel ED, VanDoorn CEA, Hessing MJC and Tielen MJM (1995). The specific stress free housing system has positive effects on productivity, health and welfare of pigs. *Journal of Animal Science*; 73:1544-1551

Evans ACO, Flynn JD, Quinn KM, Madgwick S, Crosby TF, Boland MP and Beard AP (2001a). Ovulation of aged follicles does not affect embryo quality or fertility after a 14-day progestagen estrus synchronisation protocol in ewes. *Theriogenology*; 56: 923-936

Evans ACO and O'Doherty JVO (2001b). Endocrine management factors affecting puberty in gilts. *Livestock Production Science*; 68(1): 1-12

Evans ACO, Duffy P, Crosby TF, Hawken PAR, Boland MP and Beard AP (2004). Effect of ram exposure at the end of progestagen treatment on estrus synchronisation and fertility during the breeding season in ewes. *Animal Reproduction Science*; 84 (3-4): 349-358

Evans G and Maxwell WMC (1987). *Salamons Artificial Insemination of Sheep and Goats*. Butterworths, Sydney.

Fabre-Nys C, Chesneau D, De La Riva C, Hinton MR, Locatelli A, Ohkura S, and Kendrick KM (2003). Biphasic role of dopamine on female sexual behaviour via D2 receptors in the mediobasal hypothalamus. *Neuropharmacology*; 44:354-366

Filcori M (1999). The role of luteinising hormone in folliculogenesis and ovulation induction. *Fertility and Sterility*; 71:3: 405-414

Findlay JK and Clarke IJ (1987). Journal of Reproduction and Fertility Supplement; 34:27

Findlay JK (1993) An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. *Biology of Reproduction*; 48:15–23

Fletcher IC and Lindsay DR (1968) Sensory involvement in the mating behaviour of domestic sheep. *Animal Behaviour*; 16:410-414

Fletcher IC and Lindsay DR (1971). Effect of rams on duration of oestrous behaviour in ewes. *Journal of Reproduction and Fertility*; 25:253-259

Flynn JD, Duffy P, Boland MP and Evans ACO (2000) Progestagen synchronisation in the absence of a corpus luteum results in the ovulation of a persistent follicle in cyclic lambs. *Animal Reproduction Science*; 62:4: 221-231

Forcada F, Zuniga O and Abecia J (2002). The role of nutrition in the regulation of LH secretion during anoestrus by the serotoninergenic and dopaminergenic systems in Mediterranean ewes treated with melatonin. *Theriogenology*; 58: 1303-1313

Foster DL and Ryan KD (1979). Endocrine mechanisms governing the transitions into adulthood: a marked decrease in inhibitory feedback action of oestradiol on tonic secretion of luteinising hormone in the lamb during puberty. *Endocrinology*; 105: 896-910

Fulkerson WJ, Adams NR and Gheradi PB (1981). Ability of castrate male sheep treated with oestrogen or testosterone to induce and detect oestrus in ewes. *Applied Animal Ethology*; 7:57-66

Garcia-Villar R, Schams D, Alvinerie M, Laurentie MP and Toutain PL (1984). Activity of the genital tract and plasma levels of oxytocin and cortisol at the time of mating in the ewe. *Journal of Endocrinology*; 105:323-329

Garverick HA and Smith MF (1986). Mechanisms associated with subnormal luteal function. *Journal of Animal Science*; 62 (Supplement 2) :92-105

Garverick HA, Zollers WG and Smith MF (1992). Mechanisms associated with corpus luteum lifespan in animals having normal or subnormal luteal function. Animal Reproduction Science; 28:111-124

Gelez H, Chesneau D and Fabre-Nys C (2002). Which structures are activated in the anoestrous female brain by the male or its odour? *3rd Forum of European Neuroscience, Paris*; p65

Gelez H and Fabre-Nys C (2004). The male effect in sheep and goats: a review of the respective roles of the two olfactory systems. *Hormones and Behavior*; 46:257-271.

Gelez H, Archer E, Chesneau D, Lindsay D and Fabre-Nys C (2004a). Role of sexual experience in the neuroendocrine control of ewe's sexual behaviour. *Hormones and Behavior*; 45:190-200

Gelez H, Archer E, Chesneau D, Magallon T and Fabre-Nys C (2004b). Inactivation of the olfactory amygdala prevents endocrine response to male odour in anoestrus ewes. *European Journal of Neuroscience*; 19:1581-1590

Gelez H, Archer E, Chesneau D, Campan R, Fabre-Nys C (2004c). Importance of learning in the response of ewes to male odour. *Chemical Senses*: 67:555-563

Ginther OJ, Bergfelt DR, Beg MA and Kot K (2001). Follicle selection in cattle: role of luteinising hormone. *Biology of Reproduction*;64:1:197-205

Gomez Brunet A, Lopez Sebastian A, Picazo RA, Cabellos B and Goddard S (1995). Reproductive response and LH secretion in ewes treated with melatonin implants and induced to ovulate with the ram effect. *Animal Reproduction Science*; 39:23-34

Gonzalez R, Orgeur P and Signoret JP (1988). Luteinising hormone, testosterone and cortisol responses in rams upon presentation of estrous females during the breeding season. *Theriogenology*; 30:1075-1086

Gonzalez R, Orgeur P, Poindron P and Signoret JP (1989). Seasonal variation in LH and testosterone responses of rams following the introduction of oestrous ewes. *Animal Reproduction Science*; 21:249-259

Gonzalez R, Orgeur P, Poindron P and Signoret JP (1991a). Female effect in sheep. I. The effects of sexual receptivity of females and the sexual experience of rams. *Reproduction Nutrition and Development*; 31:97-102

Gonzalez R, Levy F, Orgeur P, Poindron P and Signoret JP (1991b). Female effect in sheep. II. Role of volatile substances from the sexually receptive female; implication of sense of smell. *Reproduction Nutrition and Development*; 31:103-109

Goodman RL and Karsch FJ (1980). Pulsatile secretion of luteinising hormone: Differential suppression by ovarian steroids. *Endocrinology*; 107:1286-1290

Gordon L (1983). *Controlled Breeding of Farm Animals* (Part 2). Pergamon Press, Oxford; 10-20

Gordon I (1997). *Controlled Reproduction in Sheep and Goats*. Editor: Gordon I, Volume 2, CAB International, London.

Graham JM and Desjardins C (1980). Classical conditioning: Induction of luteinising hormone and testosterone secretion in anticipation of sexual activity. *Science*; 210:1039-1041

Gunn RG (1982). The influence of nutrition on the reproductive performance of ewes in *Sheep Production*; Ed Haresign W, Butterworths, London: 99-110

Halem HA, Cherry JA and Baum MJ (1999). Vomeronasal neuroepithelium and forebrain fos responses to male pheromones and female mice. *Journal of Neurobiology*; 39:249-263

Hawken PAR, Beard AP, O'Meara CM, Duffy P, Quinn KM, Crosby TF, Boland MP and Evans ACO (2005). The effects of ram exposure during progestagen oestrus synchronisation and time of ram introduction post progestagen withdrawal on fertility in ewes. *Theriogenology*; 63:860-971

Hayashi S and Kimura T (1978). Effects of exposure to males on sexual preference in female mice. *Animal Behaviour*; 26:290-295

Homburg R (1998). Adverse effects of luteinising hormone on fertility: fact or fantasy. *Balliere's Clinical Obstetrics and Gynaecology*; 12:4:555-563

Hulet CV, Foote WC and Blackwell RL (1965). Relationship between semen quality and fertility in the ram to fecundity in the ewe. *Journal of Reproduction and Fertility*; 21:865-869

Hughes PE (1982). Factors affecting the natural attainment of puberty in the gilt in *Control of Pig Reproduction*, Editors: DJA Cole and GR Foxcroft, Butterworth, London; 117-138

Hughes PE Pearce GP and Paterson AM (1990). Mechanisms mediating the stimulatory effects of the boar on gilt reproduction. *Journal of Reproduction and Fertility Supplement*; 40:323-341

I'Anson H and Legan SJ (1988). Changes in LH pulse frequency and serum progesterone concentrations during the transition to the breeding season. *Journal of Reproduction and Fertility*; 82:341-351

Izard MK and Vandenburgh JG (1982). The effect of bull urine on puberty and calving data in crossbred beef heifers. *Journal of Animal Science*; 55:1160-1168

Izard MK (1983). Pheromones and reproduction in domestic animals in *Pheromones and Reproduction in Mammals*. Editors: Vandenburgh JG. Academic Press, New York: 235-285

Jansen HT, Iwamoto GA and Jackson GL (1998). Central connections of the ovine olfactory bulb identified using wheat germ agglutination-conjugated horseradish peroxidase. Brain Research Bulletin; 45:27-29

Jarett LR (1984). Psychosocial and biological influences on menstruation. Psychoneuroendocrinology; 9(1): 21-28

Jonmundsson JV (1986). Sheep breeding organization and recording in Iceland. *Proceedings of the New Zealand Society for Animal Production*; 46:111-11

Karsch FJ, Legan SJ, Hauger RL and Foster DL (1977). Negative feedback action of progesterone of tonic luteinising hormone secretion in the ewe; dependence on the ovaries. *Endocrinology*; 101: 800-806

Karsch FJ (1980). Seasonal reproduction: a saga of reversible fertility. *The Physiologist*; 23:29-38

Karsch FJ, Goodman RL and Legan SJ (1980). Feedback basis of seasonal breeding: test of a hypothesis. *Journal of Reproduction and Fertility*; 58:521-535

Karsch FJ (1984). Endocrine and environmental control of oestrous cyclicity in sheep in *Reproduction in Sheep*. Supervising Editors: Lindsay DR and Pearce DT. Cambridge University Press; 12-35

Karsch FJ, Bittman EL, Foster DL, Goodman RL, Legan SJ and Robinson JE (1984). Neuroendocrine basis of seasonal reproduction. *Recent Progress in Hormone Research*; 40:185-233

Karsch FJ, Cummins JT, Thomas GB and Clarke IJ (1987). Steroid feedback inhibition of pulsatile secretion of gonadotrophin releasing hormone in the ewe. *Biology of Reproduction*: 36; 1207-1218

Karsch FJ, Dahl GE, Evans NP, Manning JM, Mayfield KP, Moenter SM and Foster DL (1993). Seasonal changes in gonadotrophin releasing hormone secretion in the ewe: alteration in response to the negative feedback action of oestradiol. *Biology of Reproduction*; 49:1377-1383

Kavaliers M, Choleris M and Colwell DD (2001). Brief exposure to female odours 'emboldens' male mice by reducing predator – induced behavioural and hormonal responses. *Hormones and Behavior*; 40(4): 497-509

Keane MG (1976). Breeding from ewe lambs. *Farm Food Research*; 7: 10-12

Keisler DH, Inskeep EK and Dailey RA (1983). First luteal tissue in ewe lambs; influence on subsequent ovarian activity and response to hysterectomy. *Journal of Animal Science*; 57:150-156

Kelley RB (1937). Studies in fertility in sheep. *Bulletin of the council for scientific and industrial research of Australia;* Bulletin 112:30-37

Kendrick KM and Baldwin BA (1987). Cells in temporal cortex of conscious sheep can respond preferentially to the sight of faces. *Science*; 236:448-550

Kendrick KM, Atkins K, Hinton MR, Broad KD, Fabre-Nys C and Keverne EB (1995). Facial and vocal discrimination in sheep. *Animal Behavior*; 1665-1676

Keverne EB (1983). Pheromonal influences on the endocrine regulation of reproduction. *Trends in Neuroscience*; 6:381-384

Khalid M, Haresign W, and Bradley DG (1998). Heart rate responses and plasma cortisol concentrations in ewes; comparison between cervical and laparoscopic artificial insemination. *Animal Science*; 66(2):383-387

Killeen ID and Caffery GJ (1982). Uterine insemination of ewes with the aid of a laparoscope. *Australian Veterinary Journal*; 59: 95

Kingsbury DL and Rawlings NC (1993). Effect of exposure to a boar on circulating concentrations of LH, FSH, cortisol an oestradiol in prepubertal gilts. *Journal of Reproduction and Fertility*; 98(1): 245-250

Knight TW (1980). Onset of mating activity in Romney ewes after short period of teasing. *New Zealand journal of Agricultural Research*; 23: 277-280

Knight TW and Lynch PR (1980). The source of ram pheromones that stimulate ovulation in the ewe. *Animal Reproduction Science*; 3:133-138.

Knight TW, Dalton DC and Hight GK (1980). Changes in the median lambing dates and lambing pattern with variation in time of joining and breed of teasers. *New Zealand journal of Agricultural Research*; 23:281-28

Knight TW, Tervit HR and Lynch PR (1983a). Effect of boar pheromones, rams wool and the presence of bucks on ovarian activity in anovular ewes early in the breeding season. *Animal Reproduction Science*; 6:129-134

Knight TW, Hall DRH and Wilson LD (1983b). Effects of teasing and nutrition on the duration of the breeding season in Romney ewes. *Proceedings of the New Zealand Society of Animal Production*; 43:7-19

Knight TW (1985). Are rams necessary for the stimulation of anoestrous ewes with oestrous ewes. *Proceedings of the New Zealand Society of Animal production*; 45:49-50

Knight TW, Ridland M and Litherland AJ (1998). Effect of prior ram-ewe contact on the ability of rams to stimulate early oestrus. *Proceedings of the New Zealand Society of Animal Production*; 58:178-180

Knights M, Baptiste QS and Lewis PE (2002). Ability of ram introduction to induce LH secretion, estrus and ovulation in fall-born ewe lambs during anoestrus. *Animal Reproduction Science*; 69(3-4): 199-209

Kohlert JC, Rowe RK and Mesiel RL (1997). Intromissive stimulation from the male increases extracellular dopamine release from fluro-gold–identified neurons within the midbrain of female hamsters. *Hormones and Behavior*; 32:143-154

Kotwica G, Franczak A, Okrasa S, Koziorowski M and Kurowicka B (2002). Effects of mating stimuli and oxytocin on plasma cortisol concentration in gilts. *Reproductive Biology*; 2 (1) 25-37

Kridli RT and Said SI (1999). Libido testing and the effect of exposing sexually naive Awassi rams to estrous ewes on sexual performance. *Small Ruminant Research*; 32 (2); 12:149-152

Langford GA, Marcus GJ, Hackett AJ, Ainsworth L, Wolynetz MS, Peters HF (1979). A comparison of fresh and frozen semen in the insemination of confined sheep. *Canadian Journal of Animal Science*; 59:685-691

Lassoued N, Khaldi N, Chemineau P, Cognie Y, Thimonier J (1997). Role of the uterus early regression of corpora luteal induced b the ram effect in seasonally anoestrous Barbarine ewes. *Reproduction Nutrition and Development*; 37; 559-571

Legan SJ, Karsch FJ and Foster DL (1977). The endocrine control of seasonal reproduction in the ewe: a marked change in response to the negative feedback action of oestradiol on luteinising hormone secretion. *Endocrinology*; 101: 818-824

Legan SJ, I'Anson H, Fitzgerald BP and Akaydin MS (1985). Importance of short luteal phases in the endocrine mechanism controlling initiation of estrous cycles in anoestrous ewes. *Endocrinology*; 117:4:1530-1536

Levy F, Locatelli A. Piketty V, Tillet T and Poindron P (1995). Involvement of the main but not accessory olfactory system in maternal behaviour of primiparous and multiparous ewes. *Physiology and Behavior*; 57:97-104

Lewis PE, Bolt DJ and Inskeep EK (1974). Pattern of luteinising hormone release in progestin treated ewes. *Journal of Animal Science*; 38:6:1204-1209

Leyva V, Buckrell BC and Walton JS (1998). Regulation of follicular activity and ovulation in ewes by exogenous progestagen. *Theriogenology*; 50:3:395-416.

Lincoln GA and Short RV (1980). Seasonal breeding: nature's contraceptive. *Recent Progress in Hormone Research*; 36:1-43

Lindsay DR, Cognie Y, Pelletier J and Signoret JP (1975). Influence of the presence of rams on the timing of ovulation and discharge of LH in ewes. *Physiology and Behaviour*; 15:423-426

Lindsay DR and Signoret JP (1980). Influence of behaviour on behaviour in reproduction. *Proceedings of the 9th International Congress on Animal Reproduction and Artificial insemination*; 1:83-92

Lindsay DR (1996). Environment and reproductive behaviour. *Animal Reproduction Science*; 42:147-150

Lobato JFP and Beilharz RG (1979). Relation of social dominance and body size to intake of supplements in grazing sheep. Lobato JFP and Beilharz RG. *Applied Animal Ethology*; 5(3): 233-239

Lucidi P, Barboni B and Mattioli M (2001). Ram induced ovulation to improve artificial insemination efficiency with frozen semen in sheep. *Theriogenology*; 55:1797-1805

Martin GB, Cognie Y, Gayerie F, Oldham CM, Poindron p, Scaramuzzi RJ and Thiery JC (1980). The hormonal responses to teasing. *Proceedings for the Australian Society for Animal Production*; 13:77-79

Martin GB, Cognie Y, Gayerie F, Oldham CM, Poindron p, Scaramuzzi RJ and Thiery JC (1980). The hormonal responses to teasing. *Proceedings for the Australian Society for Animal Production*; 13:77-79

Martin GB and Scaramuzzi RJ (1983). The induction of oestrus and ovulation in seasonally anovular ewes by exposure to rams. *Journal of Steroid Biochemistry*; 19:1C: 869-875.

Martin GB, Scaramuzzi RJ, Henstridge JD (1983a). Effects of oestradiol, progesterone and androstenedione on the pulsatile secretion of luteinizing hormone in ovariectomized ewes during spring and autumn *Journal of Endocrinology*; 96:181

Martin GB, SR, Oldham CM, Lindsay DR (1983b). Effects of progesterone on the responses of Merino ewes to the introduction of rams during anoestrus. *Australian Journal of Biological Science*; 36:4:369-378.

Martin GB, Scaramuzzi RJ, Lindsay DR (1983c). Effect of the introduction of rams during the anoestrous season on the pulsatile secretion of LH in ovariectomised ewes. *Journal of Reproduction and Fertility*; 67:47-55

Martin, GB (1984). Factors affecting the secretion of LH in the ewe. *Biological Reviews*; 59:1-45

Martin GB, Cognie Y, Schirar A, Nunes-Ribeiro A, Fabre-Nys C, Thiery JC (1985). Diurnal variation in the response of anoestrous ewes to the ram effect. *Journal of Reproduction and Fertility*; 75:275-284

Martin GB, Oldham CM, Cognie Y and Pearce DT (1986). The physiological response of anovulatory ewes to the introduction of rams- a review. *Livestock Production Science*; 15:219-247.

Martin GB, Thomas GB, Terqui M, Warner P (1987). Pulsatile LH secretion during the preovulatory surge in the ewe: experimental observations and theoretical considerations. *Journal of Reproduction Fertility and Development*; 27:1023

Martin GB, Price CA, Thiery JC, Webb R (1988). Interactions between inhibin, oestradiol and progesterone in the control of gonadotrophin secretion in the ewe. *Journal of Reproduction and Fertility*; 82:319-328

Martin GB and Thomas GB (1990). Role of communication between the hypothalamus, pituitary gland and ovary in the breeding activity of ewes in *Reproductive Physiology of Merino Sheep; Concepts and Consequences*. Editors: Oldham CM, Martin GB, Purvis IW, School of Agriculture Western Australia; 23-41

Martin GB and Walkden-Brown SW (1995). Nutritional influences on reproduction in mature male sheep and goats. *Journal of Reproduction and Fertility Supplement*; 49:437-449

Martin GB, Milton JTB, Davidson RH, Banchero Hunzicker GE, Lindsay DR, Blache D (2004). Natural methods for increasing reproductive efficiency in small ruminants. *Animal Reproduction Science*; 82-83: 231-245

Maxwell WMC (1984). Current problems and future potential of artificial insemination programmes in *Reproduction in Sheep*. Editors: Lindsay DR and Pearce DT, Cambridge University Press; 291-297

Maxwell WMC (1986). Artificial insemination of ewes with frozen thawed semen at a synchronised oestrus. 1. Effect of time of onset of oestrus, ovulation and insemination on fertility. *Animal Reproduction Science*; 10:301-308

McClintock MK (1971). Menstrual synchrony and suppression. *Nature*; 229:244-245

McClintock MK (1978). Estrous synchrony and its mediation by airborne chemical mediation (Rattus norvegicus). *Hormones and Behavior*; 10:264-276

McClintock MK (1983). Pheromonal regulation of ovarian cycle: enhancement, suppression and synchrony in *Pheromones and Reproduction in Mammals.* Editor Vandenburgh JG, Academic Press Incorporated.

McEvoy TG, Robinson JJ, Aitken RP and Robinson IS (1995). The effect of time of intrauterine insemination on the development and viability of embryos collected from superovulated ewes. *Theriogenology*;46:727-738

Merriam GR and Wachter KW (1982). Algorithms for the study of episodic hormone secretion. *American Journal of Physiology (Endocrinological Metabolism)*; 243: E310-E318

Mihm, M., Curran, N., Hyttel, P., Knight, P.G., Boland, M.P. and Roche, J.F (1999). Effect of follicle persistence on follicular fluid, oestradiol and inhibin in oocyte maturation in heifers. *Journal of Reproduction and Fertility*; 102:123-130

Minton JE, Coppinger TR, Spaeth CW and Martin LC (1991). Poor reproductive response of anoestrous Suffolk ewes to ram exposure is not due to failure to secrete luteinising hormone acutely. *Journal of Animal Science*; 69:3314-3320

Mitchell LM, King ME, Aitken RP and Wallace JM (1997). Influence of lambing date on subsequent ovarian cyclicity and ovulation rate in ewes. *Animal Science*; 65:75-81

Mitchell LM, King ME, Aitken RP and Wallace JM (1999). Ovulation, fertilisation and lambing rates and peripheral progesterone concentrations in ewes inseminated at a natural oestrus during November or February. *Journal of Reproduction and Fertility*; 115:133-140

Monje AR, Albeiro R, Schiersmann, G, Chedrese J, Carou N and Callejas SS (1992). Male effect on the post partum sexual activity of cows maintained on two nutritional levels. *Animal Reproduction Science*; 29(1-2): 145-156

Morgan PD, Arnold GW and Lindsay DR (1972). A note on the mating behaviour of ewes with various senses impaired. *Journal of Reproduction and Fertility*; 30:151-152

Murtagh JJ, Gray SJ, Lindsay DR and Oldham CM (1984a). The influence of the ram effect in 10-11month old Merino ewes on subsequent performance when introduced to rams again at 15 months. *Australian Society for Animal Production*; 15: 490-493.

Murtagh JJ, Gray SJ, Lindsay DR and Oldham CM (1984b). The effect of the presence of ram on the continuity of ovarian activity of maiden Merino ewes in Spring in *Reproduction in Sheep* Supervising Editors: Lindsay DR and Pearce DT.

Nephew KP, McClure KE, Ott KL, Dubois DH, Bazer FW, Pope WF (1991). Relationship between differences in estrous cycle duration in ewes. *Biology of Reproduction*; 44:536-539.

Ngere LO and Dzakuma JM (1975). The effect of sudden introduction of rams on oestrous pattern of tropical ewes. *Journal of Agricultural Science*; 84:263-264

Nichols DJ and Chevins PF (1981). Adrenocrotical responses and changes during the oestrous cycle in mice: effects of male presence, male urine and housing conditions. *The Journal of Endocrinology*; 91(2):263-269

Niswender GD, Juengel, JL, McGuire WJ, Belfiore CJ and Wiltbank MC (1994). Luteal function: the estrous cycle and early pregnancy *Biology of Reproduction*; 50(2):239-247

Nugent RA and Notter DR (1990). Effect of cohabitation with white-faced ewes on estrous activity of Hampshire and Suffolk ewes exposed to rams in June. *Journal of Animal Science*; 68:1513-1519

O'Callaghan DO, Donovan A, Sunderland SJ, Boland MP and Roche JF (1994). Effect of presence of male and female flock mates on reproductive activity in ewes. *Journal of Reproduction and Fertility*; 100:497-503

Oldham CM and Martin GB (1978). Stimulation of seasonally anovular Merino ewes by rams II. Premature regression of ram-induced corpora lutea. *Animal Reproduction Science*; 1:291-295

Oldham CM and Cognie Y (1980). Do ewes continue to cycle after teasing? *Proceedings of the Australian Society for Animal production*; 13:82-85

Oldham CM and Pearce DT (1983). Mechanism of the ram effect. *Proceedings of the Australian Society for Reproductive Biology*; 15:72-73

Oldham CM and Pearce DT (1984). Alternative methods for synchronisation of ewes in spring using the 'ram effect'. *Proceedings of the Australian Society of Animal Production*; 15:158-159

Oleson I (1993). Effects of cervical insemination with frozen semen on fertility and litter size in Norwegian sheep. *Livestock Production Science*; 169-184

Parsons SD and Hunter GL (1967). Effect of the ram on the duration of oestrus in the ewe. *Journal of Reproduction and Fertility*; 14: 61-70

Peacock AJ and Hughes PE (1995). The effects of daily exposure of oestrous or anoestrous gilts on the attainment of puberty in the gilt. *Animal Reproduction Science*; 40:135-142

Pearce DT, Gray SJ, Oldham CM, Wilson HR (1984). Proportion of ewes ovulating in response to ram effect. *Proceedings of the Australian Society for Animal Production*; 15:164-166

Pearce DT and Oldham CM (1983). 'Ram effect' in the breeding season. *Proceedings of the 15th Annual Conference of Australian Society for Reproductive Biology*; 15: p49

Pearce DT and Oldham CM (1984). The ram effect, its mechanism and application to the management of sheep in *Reproduction in Sheep*. Editors:, Lindsay DR and Pearce DT, Cambridge University Press: 27-34

Pearce DT, Martin GB, Oldham CM (1985). Corpora lutea with a short lifespan induced by rams in seasonally anovulatory ewes are prevented by progesterone delaying the preovulatory surge of LH. *Journal of Reproduction and Fertility*; 75:79-84

Pearce GP and Hughes PE (1987). The influence of male contact on plasma cortisol concentrations in the prepubertal gilt. *Journal of Reproduction and Fertility*; 80:417-424

Pearce GP and Oldham CM (1988). Importance of non-olfactory stimuli in mediating ram induced ovulation in the ewe. *Journal of Reproduction and Fertility*; 84:333-339

Pearce GP, Paterson AM, Hughes PE (1988). Effect of short-term elevations in plasma cortisol concentration on LH secretion in prepubertal gilts. *Journal of Reproduction and Fertility*; 83:1:413-418

Pearce GP (1992). Contact with oestrous female pigs stimulates and synchronises puberty in gilts. *Veterinary Record*; 130:318-323

Pearce GP and Patterson AM (1992). Physical contact with the boar is required for maximum stimulation of puberty in the gilt because it allows transfer of boar pheromones and not because it induces cortisol release. *Animal Reproduction Science*; 27(2-3): 209-224

Pearce GP and Pearce AN (1992). Contact with a sow in oestrous or a mature boar stimulates the onset of oestrus in weaned sows. *Veterinary Record*; 130:5-9

Peirce JW, Leigh AW, daCosta APC and Kendrick KM (2001). Human face recognition in sheep: lack of configurational coding and right hemisphere advantage. *Behavioral Processes*; 55 (1): 13-26

Pepelko WE and Clegg MT (1965). Studies of mating behaviour and some factors influencing the sexual response in the male sheep Ovis aries. *Animal Behaviour*; 13:249-258

Perkins A and Fitzgerald JA (1994). The behavioral component of the ram effect: The influence of ram sexual behaviour on the induction of estrus in anovulatory ewes. *Journal of Animal Science*; 72:51-55

Perret M and M'Barek BS (1991). Male influence on oestrous cycles in female wooly opossum (Caluromys philander). *Journal of Reproduction and Fertility*; 91(2): 557-566

Peters KE, Bergfeld EG, Cupp AS, Kojima FN, Mariscai V, Sanchez T, Wehrman ME, Grotjan HE, Hamernik DL, Kittok RJ and Kinder JE (1994). Luteinising hormone has a role in th development of fully functional corpora lutea (CL) but is not required to maintain CL function in heifers. *Biology of Reproduction*; 51 1248-1254

Pfaus JG, Kippen TE and Centeno S (2001). Conditioning and sexual behavior: A review. *Hormones and Behavior*; 40:291-321

Poindron P, Cognie Y, Gayerie F, Orgeur P, Oldham CM and Ravault JP (1980). Changes in gonadotrophins and prolactin levels in isolated (seasonally or lactationally) anovular ewes associated with ovulation caused by the introduction of rams. *Physiology and Behavior*; 25:227-236

Quadagno DM, Shubeita HE, Deck J and Francoeur D (1981). Influence of male social contacts, exercise and all female living conditions on the menstrual cycle. Psychoneuroendocrinology; 6:239-244.

Quinlaven TD and Robinson TJ (1969). Numbers of spermatozoa in the genital tract after artificial insemination of progestagen treated ewes. *Journal of Reproduction and Fertility*; 19:73-86

Raadsma HW and Edey TN (1984). Dynamics of paddock mating in conventional and intensified mating systems in *Reproduction in sheep*. Editors: Lindsay DR and Pearce DT, Cambridge University Press: 1-6

Ravindra JP and Rawlings NC (1997). Ovarian follicle dynamics in ewes during the transition from anoestrus into the breeding season. *Journal of Reproduction and Fertility*; 110:279-289

Rawlings NC, Evans AVO, Honaramooz A and Bartlewski PM (2003). Antral follicle growth and endocrine changes in pre-pubertal cattle, sheep and goats. Animal Reproduction Science; 78:259-270

Rekik M, Bryant MJ and Cunningham FJ (1991). Effects of treatment with melatonin on the response of seasonally anovular ewes to the introduction of rams. *Animal Production*; 53:2: 203-207

Rekwot PI, Ogwu D, Oyedipe EO and Sekoni VO (2001). The role of pheromones and biostimulation in animal reproduction. *Animal Reproduction Science*; 65(3-4): 157-170.

Restall BJ, Restall H, Walkden-Brown SW (1995). The induction of ovulation in anovulatory goats by oestrous females. *Animal Reproduction*; 40:299-303

Revah I and Butler WR (1996). Prolonged dominance of follicles and reduced viability of bovine oocytes. *Journal of Reproduction and Fertility*; 106:39-47

Riches JH and Watson RH (1954). The influence of the introduction of rams on the incidence of oestrus in Merino ewes. *Australian Journal of Agricultural Research*; 5:141-147

Robbins TW and Everitt BJ (1996). Neurobehavioral mechanism of reward and motivation. *Current Opinion in Neurobiology*; (6) 228-236

Robinson TJ, Moore NW, Lindsay DR, Fletcher IC and Salamon S (1970). Fertility following synchronization of oestrus in the sheep with intravaginal sponges. 1. Effects of vaginal douche, supplementary steroids, time of insemination, and numbers and dilution of spermatozoa. *Australian Journal of Agricultural Research*; 21 (5):767 – 781

Robinson JJ (1974). Intensifying ewe productivity. *Proceedings of the British Society of Animal Production*; 3: New Series, 31-40.

Roche JF, Austin EJ, Ryan M, O'Rourke M, Mihm M and Diskin MG (1999). Regulation of follicle waves to maximize fertility in cattle *Journal of Reproduction and Fertility Supplement*; 54:61-71

Romano JE, Christians CJ and Crabo BG (2000). Continuous presence of rams hastens the onset of oestrus in ewes synchronised during the breeding season. *Applied Animal Behaviour Science*; 66:1-2: 65-70

Romano JE, Fernandez Abella D and Villegas N (2001). A note on the continuous ram presence, oestrous duration and ovulation time in oestrous synchronised ewes. *Applied Animal Behaviour Science*;73:3:193-198

Rosa HJD, Juniper DT and Bryant MJ (2000). Effects of recent sexual experience and melatonin treatment of rams on plasma testosterone treatment, sexual behaviour and the ability to induce ovulation in seasonally anoestrous ewes. *Journal of Reproduction and Fertility*; 169-176

Rosa HJD and Bryant MJ (2002). The ram effect as a way of modifying the reproductive activity in the ewe: a review. *Small Ruminant Research*; 45:1-16

Rosciszewska ZE (1985). The influence of earlier mating experience of ewes on their subsequent mating behaviour and reproductive performance. *Animal Reproduction Science*; 223-229

Salamon S (1976). *Artificial insemination in sheep*. Publicity press Ltd. Chippendale, NSW; 104

Sanford LM, Palmer WM, Howland BE (1974). Influence of sexual activity on serum levels of LH and testosterone in the ram. *Canadian Journal of Animal Science*; 54:579-585

Saumande J and Humblot P (2004). The variability in the interval between estrus and ovulation in cattle and its determinants. *Animal Reproduction Science*; 85 (3-4) 171-182

Scalia F and Winans SS (1975). The differential projections of the olfactory bulb and accessory olfactory bulb in mammals. Journal of Comparative Neurology; 161:31-56

Scaramuzzi RJ, McNeilly AS, Tsonis CG, Adams NR, Baird DT, Campbell BK, Downing JA, Findlay JK, Henderson KM, Martin GB and McNatty KP (1993). A model for follicle selection and the determination of ovulation rate in the ewe, *Reproduction, Fertility and Development*; 5:459-478

Schank JC (2000). Menstrual-cycle variability and measurement: further cause for doubt. *Psychoneuroendocrinology*; 25;837-847

Schank JC (2001). Measurement and cycle variability; re-examining the case for ovarian-cycle synchrony in primates. *Behavioural Processes*;56:131-146

Schinkel PG (1954). The effect of the ram on the incidence and occurrence of oestrus in ewes. *Australian Veterinary Journal*; 30:189-195

Sevi A, Taibi L, Albenzio M, Muscio A, Dell'Aquila S and Naploitano S (2001). Behavioural, adrenal, immune and productive responses of lactating ewes to regrouping and relocation. *Journal of Animal Science*; 79:1457-1465

Shackleton DM and Shank CC (1984). A review of the social behaviour of feral and wild sheep and goats. *Journal of Animal Science*; 58:500-509

Shelton M (1960). Influence of the presence of a male goat on the initiation of oestrous cycling and ovulation in Angora does. *Journal of Animal Science*; 19:368

Sherwood NM, Chiappa SA and Fink G (1976). Immunoreactive luteinising hormone releasing factor from the pituitary stalk blood from female rats: sex steroid modulation of response to electrical stimulation of preoptic area or median eminence. Journal of Endocrinology; 70:501-511

Signoret JP, Fulkerson WJ and Lindsay DR (1982). Effectiveness of testosterone treated wethers and ewes as teasers. *Applied Animal Ethology*; 9:37

Signoret JP (1990). The influence of the ram effect on the breeding activity of ewes an its underlying physiology in *Reproductive Physiology of Merino Sheep; Concepts and Consequences.* Editors: Oldham CM, Martin GB, Purvis IW, School of Agriculture Western Australia; 59-71

Signoret JP (1991). Sexual pheromones in domestic sheep. *Journal of Steroid Biochemistry*; 39(4B): 639-645

Silverman AK (1977). Role of odours (pheromones) in mammalian mating behaviour and reproductive physiology. Bibliography of Reproduction; 29; 89-90

Skinner DC, Cilliers SD and Skinner JD (2002). Effect of ram introduction on the oestrous cycle of springbok ewes (Antidorcas marsupialis). *Reproduction*; 124:509-513

Stansfield SC, Knight PG, Al-Mauly NZ and Bryant MJ (1987). Involvement of endogenous opioid peptides in the neuroendocrine response of ewe lambs to the introduction of a ram. *Journal of Endocrinology*; 115:2:333-339

Stepien A, Shemish M and Ziecek AJ (1999). Luteinising hormone receptor kinetic and LH induced prostaglandin production throughout the oestrous cycle in the porcine endometrium. *Reproduction Nutrition and Development*; 39:1-12

Squires VR and Daws GT (1975). Leadership and dominance relationships in Merino and Border Leicester sheep. *Applied Animal Ethology*; 1(3) 263-274

Thiery JC and Martin GB (1991). Neurophysiological control of the secretion of gonadotrophin releasing hormone and luteinising hormone in sheep. A review. Reproduction Fertility and Development; 3(2): 137-173

Thomas GB, Pearce DT, Oldham CM, and Martin GB (1984). Reduction in pulse frequency by steroids: difference between Suffolk and Merino ewes. *Proceedings of the Australian Society for Reproductive Biology*; 16:95-96

Tilbrook AJ, Turner AI and Clarke IJ (2000). Effects of stress on reproduction in non-rodent mammals: the role of glucocorticoids and sex differences. *Reviews of Reproduction*; 5:105-113

Turner AI, Hemsworth PH, Hughes PE, Canny BJ and Tilbrook AJ (1998). The effect of repeated boar exposure on cortisol secretion and reproduction in gilts. *Animal Reproduction Science*; 51:143-154

Underwood EJ, Shier FL and Davenport N (1944). Studies in sheep husbandry in WA. The breeding season of Merino, crossbreed and British breed ewes in agricultural districts. *Journal of Agriculture Western Australia*:11 (Series 2):135-143

Ungerfeld R and Rubianes E (1999). Estrus response to the ram effect in Corriedale ewes primed with medroxyprogesterone acetate during the breeding season. *Small Ruminant Research*; 32: 89-91

Ungerfeld R, Pinczak A, Forsberg M and Rubianes E (2002). Ovarian responses of anoestrous ewes to the ram effect. *Canadian Journal of Animal Science*; 599-602

Urratia J, Villapando F, Meza C, Mancilla C and Ramirez B (2000). Effect of ram exposure on out of season reproductive efficiency of young maiden Merino Rambouillet ewes. *Wool Technology and Sheep Breeding*; 48

Vandenburgh JG (1967). Effect of presence of a male on the sexual maturation in female mice. *Endocrinology*; 81:345-39

van Lier E, Andersson H, Perez-Clariget R and Forsberg M (1998). Effects of administration of adrenocorticotrophin hormone (ACTH) on extragonadal progesterone levels in sheep. *Reproduction in Domestic Animals*; 33:55-59

van Vugt DA (1985). Opioid regulation of prolactin and luteinising hormone secretion. in *CRC Handbook of Pharamcologic Methodologies for the study of the Neuroendocrine System*; Editors: Steger RW and Johns AN. CRC Press: 173-184

Vinoles C, Meikle A and Forsberg M (2003). Accuracy of evaluation of ovarian structures by transrectal ultrasonography in ewes. *Animal Reproduction Science*; 80 (1-2); 69-79

Walkden-Brown SW, Restall BJ and Henniawati GE (1993). The male effect in the Australian Cashmere goats; Role of olfactory cues from the male. *Animal Reproduction Science*; 32:55-67

Walkden-Brown SW Restall SW, Norton BW and Scaramuzzi RJ (1994). The 'female effect' in Australian Cashmere goats: effects of season and quality of diet on the LH and testosterone response of bucks to oestrous does. *Journal of Reproduction and Fertility*; 100:521-531

Walkden-Brown SW, Martin GB and Restall BJ (1999). Role of male-female interaction in regulating reproduction in sheep and goats. *Journal of Reproduction and Fertility Supplement*; 52:243-257

Webb R and England BG (1982). Identification of the ovulatory follicle in the ewe: associated changes in follicular size, thecal and granulosa cell luteinising hormone receptors, antral fluid steroids, and circulating hormones during the preovulatory period. *Endocrinology*; 110:3:873-881

Weems YS, Kim L, Humphreys V, Tsuda V and Weems CW (2003). Effect of luteinizing hormone (LH), pregnancy specific protein B (PSPB) or arachidonic acid (AA) on ovine endometrium of the oestrous cycle or placental secretion of prostgalandins E (PGE_2) and $F_{2\alpha}$($PGF_{2\alpha}$) and progesterone in vitro. *Prostgalandins and other lipid mediators*; 71:1-2:55-73

Weller L and Weller A (1995). Examination of menstrual synchrony among women basketball players. *Psychoneuroendocrinology*; 20:613-622

Weller L and Weller A (1997). Menstrual variability and the measurement of menstrual synchrony. *Psychoneuroendocrinology*; 22:115-128

Whisnant CS, Havern RL and Goodman RL (1991). Endogenous opioid suppression of luteinising hormone pulse frequency and amplitude in the ewe: Hypothalamic sites of action. *Neuroendocrinology*; 54:587-593

Whitten WK (1959). Occurrence of anoestrus in mice caged in groups. Journal of *Endocrinology*; 18:102-107

Wiley TM, Cardenas H and Pope WF (1997). Effect of progesterone decline at luteolysis on the ovulatory follicles and subsequent estrous cycle length in ewes. *Animal Reproduction Science*; 46:79-87

Wilson EO and Bossert WH (1963). Chemical communication among animals. *Recent Progress in Hormone Research*; 19:673-710

Wilson CH (1992). A critical review of menstrual synchrony research. *Psychoneuroendocrinology*; 17:565-591

Woodson JC (2002). Including 'learned sexuality' in the organization of sexual behavior. *Neuroscience and Behavioural Reviews*: 26(1): 69-80

Wright IA, Rhind SM, Smith AJ and Whyte TK (1994). Female to female influence on the duration of post partum anoestrus in beef cows. *Animal Production*; 59(1):45-53

Wysocki CJ and Lepri JJ (1991). Consequence of removing the vomeronasal organ. *Journal of Steroid Biochemistry and Molecular Biology*; 39:661-669

Yellon SM and Foster DL (1985). Alternate photoperiods time puberty in the female lamb. *Endocrinology*; 116:2090-2097

Yildiz S, Uzun M, Cenesiz M, Ucar O, Kaya M and Onder F (2002). Effects of sexually activated ram or ewes on the pulsatile LH secretion in anoestrous sheep. *Acta Vet Brno*; 71:297-302

Yuthasastrakosol P, Palmer WM and Howland BE (1977). Release of LH in anoestrous ewes and cyclic ewes. *Journal of Reproduction and Fertility*; 50:319-321

Zarco L, Stabenfeldt GH, Quirke JF, Kindahl H and Bradford GE (1988). Release of prostaglandin F-2 alpha and the timing of vents associated with luteolysis in ewes with oestrous cycles of different lengths. *Journal of Reproduction and Fertility*; 83:517-526

Zarco L, Rodriguez EF, Angulo MRB, Valencia J (1995). Female to female stimulation of ovarian activity in the ewe. *Animal Reproduction Science*; 39:251-258

Ziecek AJ, Bodek G, Ciereszko R, Stepien A, Kotwica G (2001). Involvement of gonadotrophins in induction of luteolysis in pigs. *Reproductive Biology*; 1:33-50

Ziecek AJ, Bodek G, Blitek A, Kaczmarek A, Waclawik N (2004). Nongonadal LH receptors, their involvement in female reproductive function and a new applicable approach. *The Veterinary Journal*; 169(1): 75-84.

Zuniga O, Forcada F, Abecia JA (2002). The effect if melatonin implants on the response to the male effect and on the subsequent cyclicity of Rasa Aragonesa ewes implanted in April. *Animal Reproduction Science*; 72(3-4): 165-174